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Radiation and the Cell[1]

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How radiation acts on living matter is of basic biologic importance, for with this agent the biologist has been able to exert some measure of control over the destiny of cells. Indeed, with radiation he has, during the past two decades, accomplished with notable success the experimental modification of cell behavior. Cells destined to follow a particular pattern of life have been caused to manifest a changed existence—even neoplastic and malignant growth.

As we look forward to still more complete control of cell function, we may profit by stopping to take note of how radiations such as X-rays and gamma rays act to induce biologic changes. Much attention has been devoted to the atomic physics of radiobiologic reactions and to the end results obtained in living test objects, but comparatively little has been given to the whole reaction and to the various kinds of reactions. It is the purpose of this paper, therefore, to set forth as complete a picture as possible, consistent with the information now at hand, and at the same time to indicate the relationship these developments may have with our present impressions of cell activity. We shall first deal briefly with the nature of matter in general and how radiation acts to modify it, after which we shall consider at some length the question of how radiation affects living matter. Being aware that there are various elaborations of the detailed concepts of matter, we shall base our remarks on a somewhat simplified picture of the Bohr atom which is adequate for the present discussion.

MATTER IN GENERAL

All matter appears to be made up of atoms, most of which are held together in particular configurations to form molecules. Molecules, in turn, serve as building units to make up the morphological entities with which we are familiar in everyday life. Atoms are believed to be composed of a central nucleus which contains practically all of the mass and which has a positive electric charge. Further, it is believed that negative units of electricity called electrons revolve in an orbital system about the nucleus.

The simplest atom is that of hydrogen (fig. 1 (H_1^1)). Its nucleus has a mass of 1 on the atomic scale and a single charge

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equal to that of the electron, but positive in sign. This nucleus is called a proton. The normal hydrogen atom has

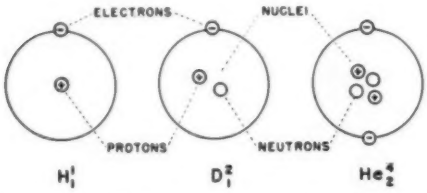

FIGURE 1.—Hydrogen, deuterium, and helium atoms.

a single orbital electron as indicated in the diagram. The net electric charge of the normal atom is zero; hence the positive charge of the nucleus must be just balanced by the negative charges of the electrons in the orbits.

Next in increasing atomic complexity after hydrogen is the atom of heavy hydrogen, or deuterium. It has a mass of 2, but only a single positive charge on the nucleus and a single orbital electron (fig. 1 (D_1^2)). Its nucleus is called a deuteron. The deuteron apparently contains an additional particle of unit mass which, however, has no electric charge—a neutron.

The element next heavier than hydrogen is helium (fig. 1 (He_2^4)). The normal helium atom has a nucleus consisting of two protons and two neutrons. It must, therefore, have two orbital electrons. The nucleus of the helium atom is an alpha particle. All other atoms appear to be made up of combinations of protons, neutrons, and perhaps alpha particles with the proper combination of orbital electrons to insure electrical neutrality.

The total weight of the nucleus, which is the sum of the weights of the protons and neutrons, is the atomic weight (expressed in relation to oxygen, 16). The number of elementary charges on the nucleus, which is equal to the number of orbital electrons, is the atomic number and defines an element chemically.

All of the elements can be placed in a series or table (the Periodic Table) in which each element has an atomic number greater by unity than the one before it. As the nuclei become more complicated and more electrons are added, the development takes place in an orderly manner. In helium, the 2 electrons occupy similar orbits. In the next substance (lithium) a second group of orbits, or shell, appears, and successive elements add an electron in this shell until it holds 8, making, with the 2 in the innermost one, a total of 10 (neon). With the next substance, sodium (atomic number 11), a third shell is added. This sort of development goes on to uranium (atomic number 92, atomic weight 238). One, and only one, element is known for every number in this series.

The chemical properties of an element are determined by the number of planetary electrons. Those in the outermost shell are the valency group. They determine the chemical affinity of atoms (or chemical radicals) for each other. Chemical union apparently is brought about by the tendency

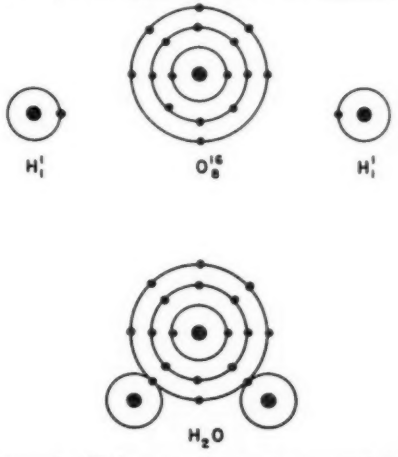

FIGURE 2.—Hydrogen and oxygen atoms and the water molecule.

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of atoms to fill their outer orbits. This, it would seem, is accomplished by some kind of common sharing of the electrons in

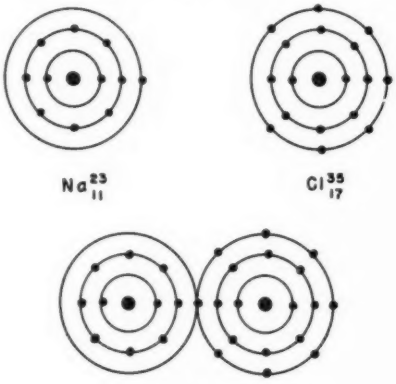


FIGURE 3.—Sodium and chlorine atoms and the sodium chloride molecule.

the outer shells of the atoms. It is clear that if a firm chemical union is effected only when outer orbits are completely filled, only certain atoms or certain groups of atoms can be combined. To illustrate, take hydrogen (H^1_1) and oxygen (O^{16}_8). Hydrogen contains one proton and one planetary electron, as shown in figures 1 and 2. Oxygen has eight neutrons and eight protons, and accordingly must have eight planetary electrons (fig. 2). Obviously, the addition of one hydrogen atom to oxygen does not furnish enough electrons to complete the oxygen's outer shell, whereas the addition of two does complete it. Two hydrogen atoms may be united with oxygen in some such arrangement as shown also in figure 2 to form the familiar water molecule. Figure 3 shows the possible arrangement of another familiar molecule (NaCl). From these examples it is clear that in terms of protons, neutrons, and electrons, large organic molecules must be exceedingly complex. Further, although the information is incomplete as to the exact manner of the electron sharing, three points important to the present development are clear: (1) Atoms tend to maintain an electrical neutrality between the nucleus and surrounding electrons; likewise, they tend to keep the innermost shells filled to capacity, as far as the number of planetary electrons will permit; (2) the chemical properties of an atom are determined by the number of planetary electrons, the valence depending on the number in the outermost shell; and (3) atoms (or radicals) are held together in groups to form molecules by the tendency to fill completely their outer shells with electrons by some kind of a common sharing of those in the valence groups.

RADIATION

The term "radiation" has come to include two quite different types of energy propagation through space—one by means of wave motion, the other by means of particles.

Electromagnetic or wave radiation includes a wide range from radio waves to gamma rays. These are not associated with matter, but represent a propagation of energy by means of waves traveling in all cases at a speed comparable with that of visible light. Particle radiation, on the other hand, comprises various atomic and subatomic particles set into very rapid motion either by the action of electric fields or of radioactive sources. The energy content of these particles depends on their mass and speed. On the other hand, when the electromagnetic radiation interacts with matter, energy is gained or lost in discrete units which may be called photons. The energy of a photon, called a quantum, is proportional to the frequency of the electromagnetic radiation. Thus, the higher the frequency or the shorter the wavelength, the greater the energy of the quantum. When electromagnetic radia-

tion interacts with matter in such a manner as to increase its energy, a wide variety of changes are produced depending upon the energy of the quantum absorbed. Since biological modifications for the most part result from chemical changes, we shall be concerned mainly with the chemical changes induced by radiation.

For convenience, in a discussion of the effects produced on matter by radiation, a unit of energy, termed the "electron volt" may be advantageously employed to convey an idea of the energy magnitudes involved. Consider for example what happens in an X-ray tube. An electron emitted from the cathode may fall through an electrical field with a potential change equivalent to the voltage applied between the electrodes of the tube before impinging on the target. If this potential difference is 200,000 volts, the energy gained by the electron is 200,000 electron volts. Electromagnetic radiation emitted by the target cannot exceed 200,000 electron volts, though it may be less if not all of the electron energy is utilized in producing a single quantum of radiation. Actually, the quanta emitted vary widely in energy content, the average being about half that of the impinging electrons or about 100,000 volts in the case cited.

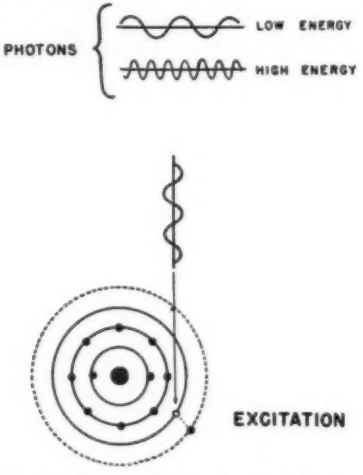


FIGURE 4.—Diagram illustrating the phenomenon of excitation in the sodium atom.

When a quantum, whose energy is equivalent to one electron volt or less, is absorbed by matter, in general the electronic structure of the atoms and molecules is unaffected, disturbances being produced of a vibrational or rotational character which result in thermal changes. So far as chemical action is concerned, only the rates of reaction which are temperature dependent are affected and not the nature of the reaction. In visible or near ultraviolet radiation where the energy of the quantum is greater than one electron volt and less than five, electronic configurations of the atom may be modified. As illustrating a change in electronic configuration, we may cite a simple case of atomic excitation (fig. 4), where a valence electron is raised to a virtual level. Such changes may result in photochemical action, though commonly the energy may be degraded into thermal disturbances and thereby affect only the rate of chemical reaction.

When a quantum with somewhat higher energy content is absorbed, the action may result in the ejection of an electron from an atom or molecule (photoelectric effect) leaving such an atom or molecule positively charged. Thus, a pair of ions is produced, one of which is positively charged and the other negatively charged. This process may give rise to chemical reaction in a variety of ways.

When the quantum of energy absorbed corresponds to that of X-rays or gamma rays (i. e., in the range of 10,000 to 1,500,000 electron volts), an electron may be ejected from an inner shell of the atom, and in addition the electron may be given a large amount of kinetic energy. Such a case of photoelectric effect is illustrated

in figure 5. Furthermore, not only may such absorption of high quantum energy remove an electron from an orbital system and give it a high kinetic energy, but also a portion of the energy may be transformed into a new photon or quantum of lower energy, this effect being known as the Compton effect (fig. 5).

Most important from the standpoint of the ultimate biological reaction is the fact that the absorption of a high energy quantum of electromagnetic radiation causes an electron to move through matter with initially high kinetic energy. This energy is lost along the electron path in the production of a multiplicity of ion pairs which leads to chemical change (fig. 6). The number of ion pairs per unit path increases as the kinetic energy of the electron diminishes, as indicated in figure 7. The energy reemitted as photons in the Compton effect may subsequently be absorbed with the production of moderately high speed electrons, which, in turn, leads to the formation of additional ion pairs. Thus, while the nature of the mechanism set in motion by high energy radiation such as X-rays and gamma rays is exceedingly complicated, it is clear that ionization plays an important part in the induction of new chemical changes. Through the gradual degradation of energy of the radiating particles, some of the terminal effects are thermal change and modification of the electronic configuration of the atoms acted upon. As indicated above the thermal change affects only the rate of chemical processes already present. However, since the thermal changes produced by biologically effective doses of X-rays or gamma rays are so small, these can have only slight, if any effect of biological significance.

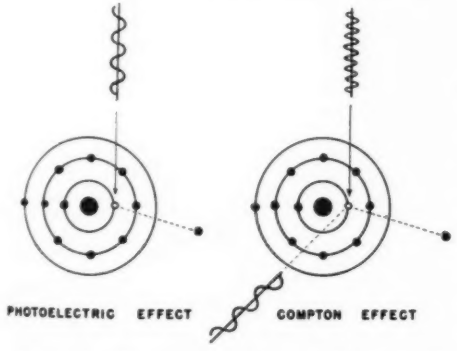


FIGURE 5.—Diagram illustrating the phenomenon of ionization by photoelectric effect and by Compton effect in the sodium atom.

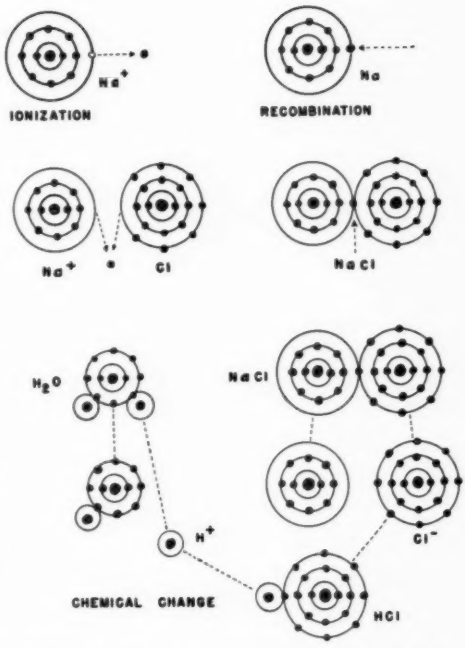


FIGURE 6.—The phenomenon of ionization and recombination in the sodium atom (at the top); ionization, the consequent molecular splitting and the eventual recombination of the sodium chloride molecule (in the middle); and molecular splitting as may be produced by ionization of atoms in the water and sodium chloride molecules with a consequent chemical change to form a hydrochloric acid molecule (at the bottom).

The changes in electronic configuration, on the other hand, do lead to new chemical processes but as yet little is known of the biological significance of such changes. However, as we shall indicate

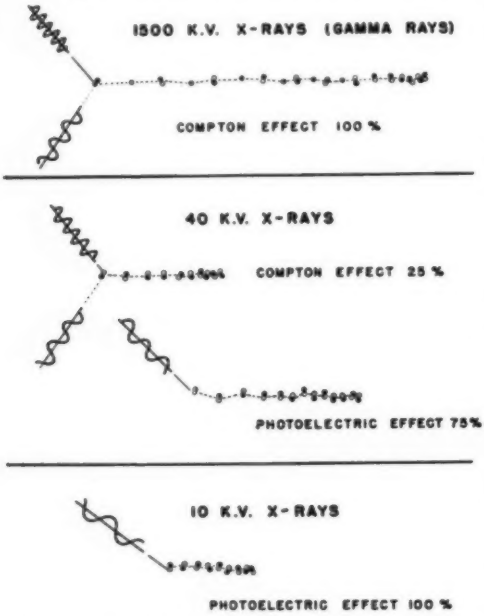


FIGURE 7.—A comparison of the ionization produced along the trajectories of recoil and photoelectrons set in motion by photons having different energy contents.

below, a good deal is known about the induction of biological changes with high energy radiation despite this gap in our knowledge.

Parenthetically, it may be stated that that biological change may result either from the destruction or loss of essential products or from the influence of newly formed photo products not previously present.

LIVING MATTER

Living matter for the most part is made up of cells—living units having usually a distinct cell membrane, a semifluid cytoplasm containing a variety of formed bodies, and a nucleus containing a matrix and certain chromatin material. These units manifest the power of growth, adaptation, and reproduction—characteristics which in a general way distinguish them as animate objects.

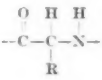
Living protoplasm is a complex system consisting of water, electrolytes, protein, and other organic compounds—enzymes, carbohydrates, lipoids, hormones, growth agents, and the like. The present concept of the cell, therefore, is that it is an exceedingly complicated system of physicochemical equilibria (acid-base, oxidation-reduction, electrolytic dissociation, permeability, etc.), which shift in orderly ways under the influence of controlling factors (chromosomes, environment, and perhaps other agents) to bring about the particular cell functions—secretion, contraction, conduction, support, proliferation, synthesis, differentiation, and the like, with which we are familiar.

Two general divisions of cell activity are thus recognized: (1) Functional, which includes all of the metabolic and nutritional activities of the cell, together with the special functions (secretion, contraction, etc.); and (2) control, which includes the regulation and direction of all cell activity.

Significant in connection with the functional activity is the fact that there must be an almost constant supply of substrate materials available together with an appreciable supply of catalysts, enzymes, and the like. This is in contrast to control activity in which there are more fixed amounts of influencing factors such as hereditary substances (chromosomes and genes), and chemical substances such as growth agents, hormones, vitamins, and special mineral substances. Thus, in cases where there is an equal opportunity for molecules of either type of material to be

injured or destroyed, the matter of availability, through concentration and replacement (by nutrition, synthesis, substitution, etc.), is likely to be of significance depending on the biologic importance of the substances involved.

Proteins are of interest both as substrate materials in metabolism and as factors in cell control. Though of wide diversity, they have one fundamental feature in common—they are composed of chains of amino acids joined in peptid linkage, each segment or link consisting of a nitrogen and two carbon atoms



These constitute the stem, or backbone, with a repeat design. One of the carbons has ketone oxygen linked with it, which is the remainder of a carboxyl group of an amino acid, whereas the other has attached to it as a sort of appendage, the amino acid residue and a side chain R, which may be a simple hydrocarbon, an alcohol, or a base. Many of these segments are added together end to end to form the protein molecule. Since the side chains allow for a wide diversity of types, the possible variety of proteins is innumerable. Attention has been drawn recently to the similarity of the chainlike (or fibrous) proteins and cell chromosomes (Waddington (*1*), Demerec (*2*)). The threadlike appearance of the extended chromosome and the two-ended nature of its chromomeres suggest that the chromosome consists of protein fibers arranged more or less parallel to its length. Geneticists, in fact, go so far as to say that chromosomes are composed of a fiberlike chromonema which is structurally similar throughout the whole chromosome complex and to which are attached various radicals; and that a segment of the chromonema with a number of radicals forms a molecular unit which is recognizable through its action as a gene. How well this most advanced concept of heredity may be fitted in with irradiation effects will be indicated later.

THE ACTION OF HIGH ENERGY RADIATION LIVING ON MATTER

Because of the discontinuous character of the action of radiation, small islands of chemical change are produced in the regions where energy quanta are absorbed. Further, because radiation such as hard X-rays and gamma rays penetrate uniformly to all parts of small living systems, these islands are produced at random in all the cell parts (membranes, cytoplasm, nuclei, chromosomes). There is at present no known mechanism by which any molecule or atom, whatever its setting, can be protected from an occasional alteration when subjected to radiations of the types in question. Hence, in an organic system that is complex to start with, many different kinds of compounds are destroyed and likewise many new ones come into existence in a chaotic fashion. Obviously, any such hodgepodge of changes would make for disorganization in cell constituents associated with either functional or control activities. But, as indicated, the constituents in the two cases differ with respect to the amount that must be changed in order to cause significant differences.

Since substrate material is available for metabolic activity in appreciable, if not excess, amounts, it is to be presumed that functional activity would not be easily affected by radiation through destruction of substrate material. In any case, it appears that cells would not suffer permanent injury from such change. Likewise, it appears that many other elements associated with functional activity such as enzymes; catalysts; formed components such as

membranes, mitochondria, cytoplasmic granules, etc., could suffer the loss of many isolated molecules without serious injury to the cell. Certain activity might be altered, but with continued replacement, the cell would soon recover and again be normal. Actually, experimental findings for the most part bear out this generalization.

Scott (*3*) in a recent article brings together the literature dealing with changes in functional activity induced by radiation. Such processes as respiration, glycolysis, nitrogenous metabolism, enzymatic activity, etc., have been investigated. In all cases cited, except two,[2] these processes were found to be little influenced even by large doses of radiation. We may thus eliminate from consideration this rather large class of reactions since it seems to be of lesser importance as far as radiobiologic reactions are concerned. It is not to be implied from this, however, that important biologic changes cannot be produced in the functional activity of cells, but rather that for doses sufficiently large to cause changes in control activity of far-reaching biologic importance, functional activity is usually affected very little.

As mentioned, control activity in cells is attended by more fixed amounts of influencing factors. Hereditary substances are made up of particularly small amounts of highly organized materials quite specifically localized along the chromosomes. Hormones and growth agents are less localized and are not in such small amounts, but the influence they exert on biologic activity depends upon specific concentrations. Thus loss of small amounts of either genetic or growth-agent materials may have profound biologic effects, and one begins to be aware of a reason why control activities of the cell are more readily modified by radiation than are the functional. Further insight into the nature of radiobiologic reactions perhaps can best be gained by considering the possible kinds of effects produced by radiation in homogeneous populations of cells.

[2] *Mottram (4) and Crabtree and Cramer (5). Scott, however, points out some reasons for believing that these findings may not constitute exceptions.*

KINDS OF RADIOBIOLOGIC RESPONSES

When a homogeneous population of cells is irradiated with X-rays or gamma rays and the percentage of cells killed is plotted graphically as a function of the dose administered, curves such as those shown in figure 8 may be obtained. Such curves have been derived through experimentation with a variety of kinds of cells and test organisms and they range in shape from those which display a marked S-shape or skew character (curve A) to those which are of the die-away type (curve D). Obviously, such curves represent some kind of final reckoning of all the factors which act to bring about the end result, and an analysis of the different types of curves should lead to some interesting facts about the reactions involved.

At the outset, one is impressed by the simple character of the curves. They are not complicated by a series of inflections with varying ranges and amplitudes.

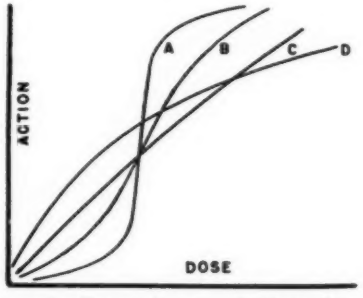


FIGURE 8.—Dose-action curves illustrating the progress of different types of radiobiologic responses.

This in itself suggests that the reactions must depend upon a fairly homogeneous change produced by the radiation and that a close relationship must exist between the production of this change and the manifestation of the lethal effect.

With this in mind, we should like to postulate the various radiobiologic reactions that might be expected, taking into account various types of cell responses and presuming that a close relationship does exist between some particular initial effect produced and the final result obtained. At the same time we should like to present examples of responses which appear to be typical of the types postulated.

TYPE RESPONSE I. SINGLE-EVENT ACTION

The simplest conceivable kind of radiobiologic response is that in which the final result is produced by a single event, that is, one in which the change produced is always holistic in each cell and the result of the single event. Evidence that such reactions occur is suggested by the fact that what appears to be exponential dose-action curves like C and D, figure 8, have been obtained in actual experiment. For example, if varying doses of X-rays are applied to uniform samples of bacteria and a count made of those that survive to form colonies on an agar plate, a curve showing the number killed as a function of the dose of radiation applied is obtained which resembles curve D (Wyckoff (*6*), Lea et al. (*7*), Lorenz and Henshaw (*8*)). A second, and perhaps most important example, is the production of mutations (mostly in *Drosophila*). If radiation is applied to the sperm of adult males which are then allowed to inseminate normal females, a higher percentage of mutations will be found among the progeny of such individuals, and the quantitative relationship between dose of radiation administered and number of mutations produced gives rise to a curve resembling curve C.[3]

In these two cases, the curves are alike in that they appear to have no threshold part; that is, no region at the beginning where little or no effect is manifest. It thus appears that no accumulation of events must occur before the effect begins to appear, a finding which indicates that the successful registration of a single event in a particular sensitive region is sufficient to cause cell death. The die-away character of the curves (more particularly in curve *D*) indicates that for equal increments of radiation dosage, fewer and fewer organisms are killed as the reaction progresses. This is in accord with what would be expected on the basis of purely random action, since the chances of the successful encounter of an energy unit with a sensitive cell part drops off as the number of viable cells diminishes. Such a reaction, if not complicated by other processes, would proceed in accordance with the law of mass action, and the die-away curve would be strictly of the exponential type. The experimental curves obtained appear to be of this character. Let us therefore accept single-event action as a possible type for purposes of classification, leaving the questions of plausibility to be dealt with later.

TYPE RESPONSE II. MULTIPLE-EVENT ACTION; EFFECT MANIFEST IN HOLISTIC FASHION

In contrast to single-event action is multiple-event action—that in which an effect is manifest after an accumulation of events in the living test object. By deduction it becomes apparent that the dose-action curve will have different shapes and different meanings depending on whether the response is holistic in character or manifest by degree in each

[3] *For recent summaries of this work see Patterson and Muller (9), Schultz (10), and Timofeeff-Ressovsky (11).*

cell as the events accumulate. Here we shall deal with the former.

For this, experiments of our own on the production of multipolar cleavage in the eggs of the sea urchin, *Arbacia punctulata*, furnish an excellent example. If adequate doses of X-rays (around 20,000 r.) are administered to sperm (or eggs or both) before fertilization, the first cleavage of the egg, when it appears, will be multipolar; that is, instead of the egg dividing into two equal blastomeres, as is normal, it divides into more than two. The curve showing how the number of organisms undergoing this type of change varies with the dose of radiation applied resembles curve *A*, figure 8. This curve is S-shaped, thus displaying a significant threshold, a measure of which, when quantitatively expressed, gives an indication of the amount of radiation which must be given before the effect begins to be produced. The characteristic features of this reaction are that it is accumulative, and that the effect, if it appears at all, is manifest completely.

TYPE RESPONSE III. MULTIPLE-EVENT ACTION; EFFECT MANIFEST BY DEGREE

Interestingly, a graded type of response was observed in the same sea-urchin material treated in the same way. If X-rays are applied to sperm which are then allowed to fertilize normal eggs and the eggs watched, it will be found that the interval between the moment of insemination and the time when 50 percent of the eggs have divided is definitely prolonged. Further, as the amount of exposure to X-rays is increased, the amount of delay is likewise increased. The curve obtained, showing how the amount of delay varies with exposure of the sperm to X-rays, has no threshold part and is of the die-away type. Thus, whereas it resembles curve D, it obviously means something quite different here. In this case, the effect in each cell varies with the amount of radiation applied. The reaction is thus of the multiple-event type, each event contributing to the amount of effect in each test unit. Whereas the exponential character of the curve in the former case suggested single-event action, here it suggests that a single substance or a single homogeneous condition is being modified progressively in each cell.

These are the basic type responses visualized, as allowed by the assumption that the effect observed gives a direct measure of the initial irradiation effect produced, and also that a uniform type of response is dealt with. The first type of action implies that the biologic change, whatever its nature, is caused by a single event; the second, that a number of such events are required, but that the effect, when manifest, becomes so completely; and the third, that each event contributes quantitatively to the amount of effect observed in each cell, whatever the nature of this effect may be.

Varied responses—those not uniform in character—may also be produced. Whereas one may select just one kind of change and study it to the exclusion of the rest and deal with it under the categories listed above, it sometimes aids in the analysis to deal with the variety of changes. In the present development such a consideration is especially suggestive. Two examples of the varied type of response will be given.

Radiation applied to frog's sperm before fertilization of normal eggs, causes larvae to form which show a wide variety of abnormalities. To mention only a few, some individuals show dorsal flexure of the tail, some lateral flexure, and still others ventral flexure; some show a marked edema while others show none; some show spina bifida and various degrees of twinning while others do not. It is thus clear that

the factors present in the sperm having to do with later development in the egg are affected in different ways, and further that any one of a variety of abnormalities produced may lead to death of the organisms.

The second example has to do with the killing action of X-rays on free-living cells of yeast (*Torula cremoris*). If varying amounts of radiation are administered to identical samples of these cells which are then put to culture on agar plates, it is found that the number of colonies which will appear diminishes as exposure to the radiation increases. In fact, what appears to be an exponential-type killing curve is obtained. In accord with what has been said, this leads to the presumption that single-event killing action is in effect here and that when the lethal blow has been struck, the cell fails to give rise to a colony. While this may be and probably is what happens, there is this interesting additional aspect: If one examines microscopically the cells which fail to give rise to colonies visible to the unaided eye, it is found that some cells do not divide at all after treatment, that others divide once, others twice, three, four, and some a good many times before succumbing to the irradiation injury. It is, therefore, apparent that death is not brought about the same way in each organism. Here, as in the case of the frog's sperm, it seems that the cells must contain a variety of sensitive regions, injury to any one of which may lead to death. This, in fact, seems quite plausible if we accept the advanced concepts of hereditary materials as described and also the mechanism of the induction of chemical change as set forth. Since hereditary elements or genes are located along the body of chromosomes like beads on a string, and since these may also be special side radicals of long chainlike molecules, it is easy to visualize how radiation quanta acting at random to split molecules may dislodge or dislocate one combination of factors in one case and quite a different combination in another. Thus, even though the same mechanism of action might be involved, quite different results would be obtained or the same result (such as death) obtained by different vital genes being destroyed or removed.

When varied types of responses, such as these, are encountered, they may be dealt with under the categories given above by doing one of two things: (1) Selecting a particular kind of effect and dealing with it to the exclusion of all the rest; or (2) dealing with part or all of the changes as a homogeneous group. It will be recognized that it was the latter procedure which was used in connection with the yeast experiments and that doing so made possible the interesting correlation between the single-event action suggested by the dose-action curve and the idea that death is produced by injury to any one of a variety of sensitive regions as indicated by the microscopical examinations.

It is thus apparent that responses have been obtained through experimentation which seem to typify each of the kinds of reaction postulated. While this is interesting, it can have no particular significance until the basic assumptions are examined for plausibility.

PLAUSIBILITY OF BASIC ASSUMPTIONS

It will be recalled that the classification as just outlined is based on the supposition that a close relationship exists between the irradiation effect observed and the atomic changes produced. It is necessary at this point, therefore, to inquire just how close this relationship is.

It has been emphasized repeatedly throughout this development that when living systems are exposed to radiation, no cell constituent can be excluded from the possibility of alteration. Our question

squarely put, therefore, is whether in the vast array of changes produced in a cell there is a particular change or particular class of changes which leads directly to the end result under consideration.

Perhaps the strongest indirect evidence that there is such a close relationship is the fact that reactions have actually been observed resembling all of the different types that can be postulated. By referring to type response III, it appears that because of the unique character of the response, the curve can have no other meaning than that indicating some change induced directly by the radiation which varies progressively in each cell. By referring to type response II, it is also clear that irrespective of what interpretation is placed on the nature of the reaction, a threshold relationship exists between the effect produced and the dose administered. Thus, so far as the existence of these features as realities is concerned, there can be little doubt; and since they form integral parts of the basic concepts of the postulation, the experimental findings may be taken as evidence supporting the postulation as a reality.

Whether cell changes, extensive as cell death, can result from a single event as suggested by type response I, however, is quite another matter. Could it be proved beyond doubt that the dose-action curve in this case is actually exponential in character, it would be difficult, if not impossible, to offer any other interpretation. Fano, in a report now in press, points out the extreme difficulty one may have deciding whether the best curve which fits experimental data, with no more than the usual variation, is of the single- or multiple-event type. Yet on the outcome of such decisions rests a large part of the support for the idea of primary random action. In the light of Fano's findings, it seems proper to conclude, therefore, that whereas the quantitative data do not disprove that the curves are exponential, neither do they furnish really strong proof that they are of this type.

Fortunately, however, there is further information apart from that of the dose-action curves which lends support to the idea of a close relationship between initial effect and final result. First, there is the now abundant evidence that mutations (including the lethal variety) may result from chromosome aberration (deletion, inversion, translocation), and the fact that such aberrations are induced by radiation. In addition, there is the evidence that these changes are produced independently of the duration of exposure, of temperature during treatment, and of the metabolic activity during treatment. Mutations, by definition, are all-or-none in character; and, as indicated above, the dose-action curve for their production appears to lack completely any threshold character. Now, should it be supposed that chromosome aberration is induced by secondary influence—say, by some secondary chemical change in the cell, the temperature, growth rate, metabolic activity, etc., would be expected to modify the quantitative results obtained. Since, however, experimental findings indicate that they do not, such findings may be taken as evidence in support of primary random action.

Perhaps the strongest support yet available is that obtained by Sax (*12*) which combines indirect and direct evidence. Sax noted that if certain plant cells (*Tradescantia*) are irradiated during the resting stage when the chromatin material is in the form of long, single threads, the chromosomes upon subsequent examination are actually seen to be broken or fragmented, thus giving direct evidence of a discontinuous type of action. If, then, a plot is made of the percentage of cells containing single chromosome breaks as a function of the

dose of radiation applied, a nonthreshold type curve is obtained. On the other hand if a plot is made of the percentage of cells containing two breaks, a definite threshold-type curve is obtained. Since the quantitative data supporting these findings were obtained under especially favorable conditions, and since the findings are strictly in accord with the predictions based on the idea of chromosome breaks being produced by single action, they may be taken as strong evidence substantiating the idea of single-event action.

Up to this point, nothing has been said about the nature of the event, that is, as to whether it consists of the production of a single ion pair, the passage of an electron, or the absorption of the total energy of a photon. The simplest conceivable kind of reaction is one produced as a result of a single ion pair being formed. From that which has been said above about molecular change being induced by the removal of a single electron and about the possible nature of chromosomes, there is reason to believe that biological change extensive as death may result from an event as simple as the production of a single ion pair. It was indicated that removal of an electron by ionization of a molecule may cause that molecule to split and undergo chemical change. Hence, in view of the fact that hereditary elements may consist of long chains of protein-like fibers, it may be presumed that an ionization at various points along the body of a chromosome may cause a break which would result in a deletion, or, in the case of additional breaks, in inversion or translocation. Thus, whereas photon absorption and electron passage may act as unit events to produce biological changes, it seems quite possible that such changes may also result from single ion-pair formation.

SUMMARY

1. A substantial body of evidence has been assembled in support of the concept that a close, if not direct, relationship exists between certain radiobiologic results observed and the initial irradiation effect produced.

2. The exponential dose-action curves, insofar as they are reliable, may be taken to indicate that biologic changes extensive as cell death may be induced by single-event action—perhaps even by the production of single ion pairs.

3. Because of our work with sea urchin gametes, we have been able to deal with the quantum aspects when the effect is of the accumulative type in each cell. In considering the inferences which come as a result, our attention was attracted to the question of what types of response could be postulated when a uniform type of response is dealt with and when a direct relation exists between the initial effect produced and the effect observed. The following were visualized:

Type response I. Single-event action.

Type response II. Multiple-event action; effect manifest in holistic fashion.

Type response III. Multiple-event action; effect manifest by degree.

Experimental findings are presented which appear to fit perfectly into each of these categories and reasons are advanced for believing that a large portion of the direct cell responses to radiation falls naturally into these divisions.

4. Accepting the idea of random event, what does all this mean to biology?

First of all, it directs attention to the possibility of discontinuity reactions in cells; that is, to reactions in which isolated single events may produce biologic changes of far-reaching importance.

Secondly, it indicates the uniqueness of

certain entities in cells, that these entities are of molecular dimension, and that single entities of this type may in a large measure control the destiny of cells.

Thirdly, and most important, the irradiation technique provides a means of performing surgical operations on such entities with a degree of ultramicroscopic fineness and at the same time provides a means of studying their nature and behavior.

5. Finally, mention should be made that the above considerations pertain only to free-living cells, whereas the responses of cell groups or tissues comprise quite a different chapter which likewise must be taken into account when formulating rationals of radiotherapy or biologic studies.

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Studies in Carcinogenesis

XIII. Tumors of the Spleen and Liver in Mice Following the Introduction of Hydrocarbons Into These Organs[1]

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INTRODUCTION

The results obtained in the study reported in this paper, in conjunction with those in the literature on the production of malignant tumors of the spleen and of the liver by the introduction of carcinogens directly into these organs, indicate that the liver and spleen are less susceptible to tumor induction than are the subcutaneous tissues.

As regards direct injection into the spleen, Ilfeld (*1*), using Shear's (*2*) cholesterol-hydrocarbon technique, did not obtain any tumors of the spleen in 56 animals (50 mice and 6 rats) with pellets that contained 5 percent of 1,2,5,6-dibenzanthracene [3]; with pellets containing 5 percent of 20-methylcholanthrene [3] he obtained a sarcoma of the spleen in 1 mouse (2 animals injected; 1 mouse and 1 rat). Furth and Furth (*3*) obtained splenic sarcomas in 2 of 96 mice, of 5 different strains, with 0.05 cc. of a 4-percent solution of 3,4-benzpyrene [3] in lard; the 2 cases of hemangioma of the liver and spleen which occurred in their mice were not definitely classified as induced or spontaneous. Rusch, Baumann, and Maison (*4*) obtained, with dibenzanthracene, a sarcoma of the spleen in 1 of 11 animals (9 heterozygous rats and 2 male C_3H mice). With benzpyrene they did not obtain tumors of the spleen in any of 11 animals (2 heterozygous rats and 9 male strain A mice). These workers gave doses of hydrocarbons ranging between 0.25 and 4 mg., dissolved in corn oil.

In the introduction of hydrocarbons directly into the liver, Ilfeld (*1*) employed cholesterol-hydrocarbon pellets in 64 animals (56 mice of 3 inbred strains, 7 rats, and 1 heterozygous mouse). The pellets contained 5 percent of dibenzanthracene, of benzpyrene, or of methylcholanthrene. The only tumors of the liver that were obtained occurred in 2 mice of the C_3H strain. Ilfeld's gross and microscopic descriptions of these tumors are consistent with those of the spontaneous hepatomas which occur in a considerable percentage of C_3H mice; since the latter fact was not recognized at the time of Ilfeld's work, it is open to question that this author was dealing with induced tumors. Oberling and others (*5*) injected a colloidal solution of benzpyrene into the liver in 5 rats and 5 fowls. None of the rats lived longer than 8 months, and 3 of the fowls were still alive after 10 months; no tumors were observed in the livers of any of these animals. Woglom (*6*) inserted cotton threads impregnated with benzpyrene in various

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[3] *Hereafter referred to as dibenzanthracene, methylcholanthrene, and benzpyrene, respectively, for brevity.*

tissues of a large number of rats. Although this technique produced tumors when the threads were placed in the subcutaneous tissues, no tumors of the liver were obtained in the rats with intrahepatic implantation of threads; similarly negative results were obtained with mice.[4] Oberling, Guérin, and Guérin (7) implanted crystals of benzpyrene in the livers of 10 white rats; sarcoma of the liver was noted in 2 animals after 16 and 25 months, respectively. Rusch, Baumann, and Maison (4), using 0.25 to 2.6 mg. dibenzanthracene in corn oil, obtained no tumors of the liver in any of 12 animals (9 heterozygous rats and 3 male C_3H mice). Esmarch (8) injected 8 mg. methylcholanthrene, in "fat," into the livers of 10 Bagg albino mice. The experiment lasted for 7 months; no hepatic tumors were induced.

Thus the literature reveals the production of only 4 splenic tumors in 176 mice and rats, and of only 2 hepatic tumors in more than 100 mice and rats, by direct introduction of hydrocarbons into these organs. It is apparent that, with the techniques hitherto employed, tumors of these organs have not been readily induced in either mice or rats. It furthermore appears that implantation in the liver has so far not given rise to even a single indubitably induced tumor in mice.

In an earlier study,[5] cholesterol pellets containing methylcholanthrene were implanted in the liver or in the spleen of 120 rats with essentially negative results as regards tumor production. Because of the ease with which tumors are produced by carcinogenic hydrocarbons in many organs and tissues, this refractivity on the part of the liver was unexpected. Further experiments were thereupon carried out with mice. Carcinogenic hydrocarbons were introduced, by a variety of procedures, into the livers of 53 mice of several strains, with essentially negative results in most cases and with equivocal results in a few cases. Methylcholanthrene pellets were implanted intrasplenically in 20 mice; splenic tumors were obtained[6] in 5 of the 12 animals that lived for more than 7 months, a higher proportion than hitherto reported as having been produced by hydrocarbons.

METHODS AND MATERIALS

The hydrocarbons employed had the melting points described in Fieser's review (11). The mice, all of inbred strains, were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The animals were fed a diet of Purina dog chow supplemented with white bread and milk for a few weeks after their arrival at the laboratory. Thereafter they received only the dog chow. Tap water was offered freely at all times. Laparotomy was performed in these mice for exposing the spleen and the liver.

[4] *The number of rats employed in the experiment with the liver was not stated in this brief report; 497 rats were used for experiments on 5 different tissues. No details of the mouse experiments were given.*

[5] *Jones, B. F., Rothman, A. J., and Shear, M. J.: Unpublished experiments. (1935.) The rats were of a hooded buff stock of the Fearing Laboratory of the Free Hospital for Women, Brookline, Mass., where these experiments were carried out. Some of the rats, both in the test and control groups, developed ulcerative cecitis, a disease occurring spontaneously in the rats of this colony. A study of this disease has since been made by Jones and Stewart (9).*

The pellets contained 5 and 10 percent methylcholanthrene, respectively. In 10 rats the pellets were introduced intrasplenically, and in 110 rats they were implanted in the liver. Of these latter animals the liver was left intact in some; in others a large part of the liver had been excised, prior to implantation of the pellet, with the expectation that the regeneration of the liver tissue might contribute to tumor production. The pellets were recovered intact from the liver in most cases: in a small number, however, the pellets were much reduced in size, indicating partial absorption. Induction of tumors of the spleen or of the liver was not definitely established, although the experiment lasted for about 20 months. No tumors developed in the livers as a result of the experimental procedure. However, localized cirrhosis was noted in 1 rat, and small areas of proliferation of atypical spindle cells occurred around the pellets in 3 others.

[6] *A preliminary report (10) of the production of these tumors of the spleen was made at the Baltimore meeting of the Federation of American Societies for Experimental Biology, April 1, 1938.*

For splenic implantation, a small incision was made in the lateral surface of the spleen near the inferior pole. A methylcholanthrene pellet weighing 1.5 mg., as previously prepared (*12*), was introduced through the incision; hemorrhage was controlled by application of absorbent cotton.

For introduction into the liver, several techniques were employed. In one of the experiments a cotton thread coated (*13*) with methylcholanthrene, benzpyrene, or dibenzanthracene, was stitched into the liver. In coating the thread, its tip was dipped into molten hydrocarbon. When cool, it was stitched through the liver until the heavily coated tip was completely buried in hepatic tissue; the thread was then cut off flush with the surface of the liver, leaving the tip implanted. In another experiment a piece of wool yarn, after being soaked in a warm lard solution [7] containing 4 mg. methylcholanthrene per cubic centimeter, was sutured through the liver and tied so that the loop passed through and embraced one lobe. In a third experiment, 0.03 to 0.05 cc. of a solution of methylcholanthrene in light mineral oil was injected directly into the liver. The solution, which contained 10 mg. of the hydrocarbon per cubic centimeter, was clear at elevated temperatures but deposited crystals on cooling. In the fourth experiment a 1.5-mg. methylcholanthrene pellet (*12*) was implanted by nicking the capsule and forcing the pellet well into the liver tissue.

The tissues removed at autopsy were fixed in U. S. P. formalin diluted 1 to 10, and blocked in paraffin. The stains employed were haematoxylin-eosin, van Gieson's stain for collagen, and Foot's modification of Bielschowsky's reticulum stain.

[7] *The lard employed was the fraction liquid at 38° C.*

RESULTS

INTRASPLENIC IMPLANTATION

A methylcholanthrene pellet was implanted (March 30, 1937) intrasplenically in 20 young male C_3H mice. Of the 12 animals which survived for more than 7 months, splenic tumors developed in 5; in another mouse there was a spindle-cell sarcoma in the upper anterior abdomen, not in contact with the spleen. These 12 mice survived for the following periods: 7.5 months (2 mice), 8 months (4 mice), 10 months (2 mice), 11 months (1 mouse), 13 months (2 mice), 15 months (1 mouse). The 5 mice with tumors of the spleen died or were killed 7.5, 8, 10, 10, and 15 months after implantation of the pellet.

The pellets were seen in the spleen at autopsy in 4 of the 8 mice that died before 7 months, and in 8 of the 12 mice that lived beyond that time, i. e., the pellets were observed at autopsy in 12 of the 20 mice. In most of the animals without tumor, little or no gross change was noted in the splenic tissue immediately in contact with the pellet. In 1, however, the pellet was surrounded by a dark red zone 2 mm. in diameter forming a localized globular enlargement of the spleen in this area. In 4 cases there was diffuse amyloidosis of the spleen.

Of the five splenic tumors, four were confined to the spleen, and the fifth infiltrated adjacent structures. The tumors were irregular in shape with an average diameter of 10 to 20 mm. The tumor tissue was soft or firm, and gray or pink, with yellow and red mottling in some areas. The pellet was found in the tumor in three cases (fig. 1). Three of the five tumors were located at the inferior pole of the spleen at the injection site. The fourth tumor involved the spleen more extensively (fig. 2); the pellet was present in the interior of the tumor.

The fifth tumor replaced almost the entire spleen and infiltrated the stomach, pancreas, mesentery, and abdominal wall.

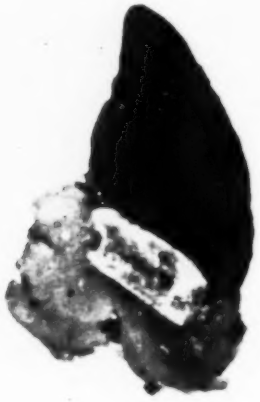

FIGURE 1.—Cross section of spleen and of splenic tumor (A 1199) from a C_3H mouse. The methylcholanthrene pellet is present, partly imbedded in the tumor, and partly in contact with splenic tissue. Gross photograph ×10.

There were extensive peritoneal adhesions, and the wall of the distal half of the stomach was markedly thickened by tumor tissue.

Microscopic examination revealed that all five tumors were spindle-cell sarcomas with the cells arranged in interlacing bundles. In two tumors the cells and nuclei were fairly regular. In the other three, while most of the cells were spindle-shaped, there was wide variation in size, shape, and staining; the tumors contained round, oval, giant, plump, markedly elongated, pale, and hyperchromatic cells. The spindle-shaped cells frequently tapered off into fine fibrils. The giant cells had single or multiple heavy walled nuclei, sometimes lobated, with large chromatin masses. Mitotic figures varied in frequency from one to several per three high-power fields. In all the tumors there was an abundance of reticulum forming a fine meshwork in intimate contact with individual cells or small groups of cells. The amount of collagen present varied from tumor to tumor, and from area to area in the same tumor. It was most abundant about the blood vessels and in the necrotic and hemorrhagic areas. The collagen in the interlacing bundles was moderate in amount in three cases, slight in one, and almost totally absent in another. In four tumors the blood vessels were numerous; in one of these they were dilated and congested, giving the tumor an angiomatous appearance. The blood vessels were all of the immature type; many were slitlike and thin-walled, and in some instances no wall was discernible separating blood from the tumor cells. There were large

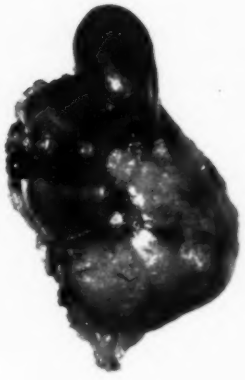

FIGURE 2.—Spleen (A 1232) of a C_3H mouse with large induced tumor replacing most of the organ. Gross photograph ×3.

areas of hemorrhage and necrosis in two tumors, in one of which there were numerous small foci of myxomatous degeneration. One tumor was extensively

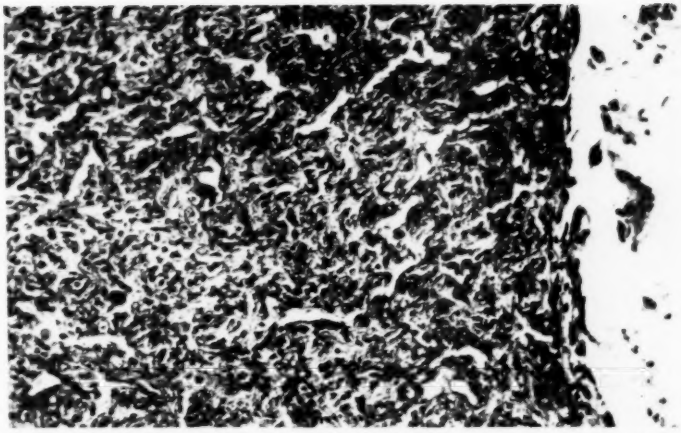

FIGURE 3.—Sarcoma of spleen (A 1232) of a C_3H mouse. The space occupied by the methylcholanthrene pellet (at right) is bordered by tumor tissue which was in direct contact with the pellet. Photomicrograph ×225.

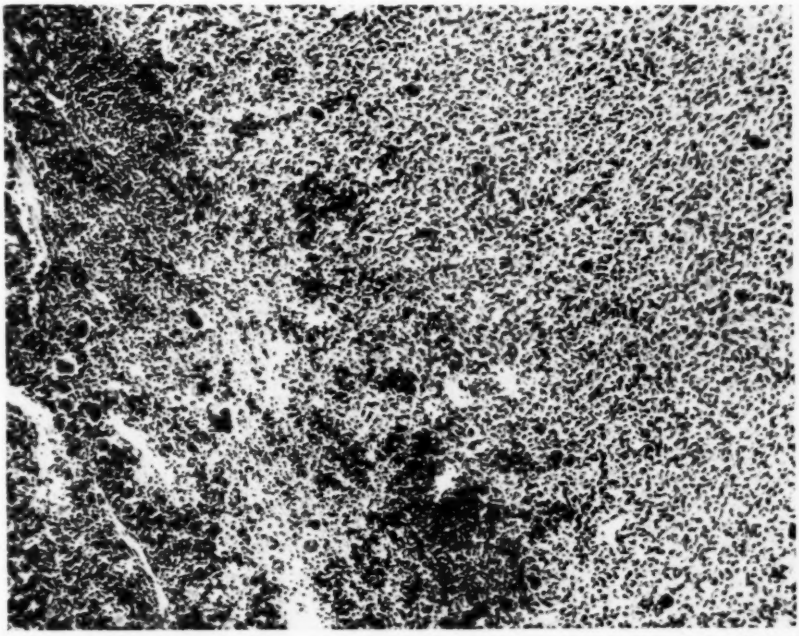

FIGURE 4.—Spleen (A 1735) of a C_3H mouse with spindle-cell sarcoma induced by a methylcholanthrene pellet. The tumor tissue invades the splenic tissue, and infiltrates and separates the cellular and fibrous elements of the organ. Photomicrograph ×112.

infiltrated with myeloid cells. The pellet space in another contained a few necrotic cells and acidophilic granular material; the viable tumor cells were observed in direct contact with the pellet space (fig. 3). In all cases a portion of the spleen with the tumor attached was studied. The tumor tissue showed direct invasion of the splenic tissue, infiltrating and separating the cellular and fibrous elements of the organ (fig. 4).

One of the splenic tumors was transplanted for three generations in the subcutaneous tissues of other C_3H mice before passage was discontinued. The transplants retained the characteristics of the primary sarcoma.

In the mice which did not develop tumors, the pellet space in the spleen was surrounded by a thin capsule of hyalinized connective tissue containing a few inflammatory cells. In two cases there was, in addition, an area of atypical hyperchromatic spindle cells adjacent to the capsule; the larger area measured 2 mm. in diameter. The spindle cells showed occasional mitotic figures and a tendency to be arranged in interlacing bundles containing a fine meshwork of reticulum, a few collagen strands, and immature blood vessels (fig. 5). These lesions were not fully enough developed to be designated as sarcoma, but were suggestive of early neoplastic transformation.

No sarcomas of the spleen, of the type described here, have been observed to have occurred spontaneously in mice in this laboratory.

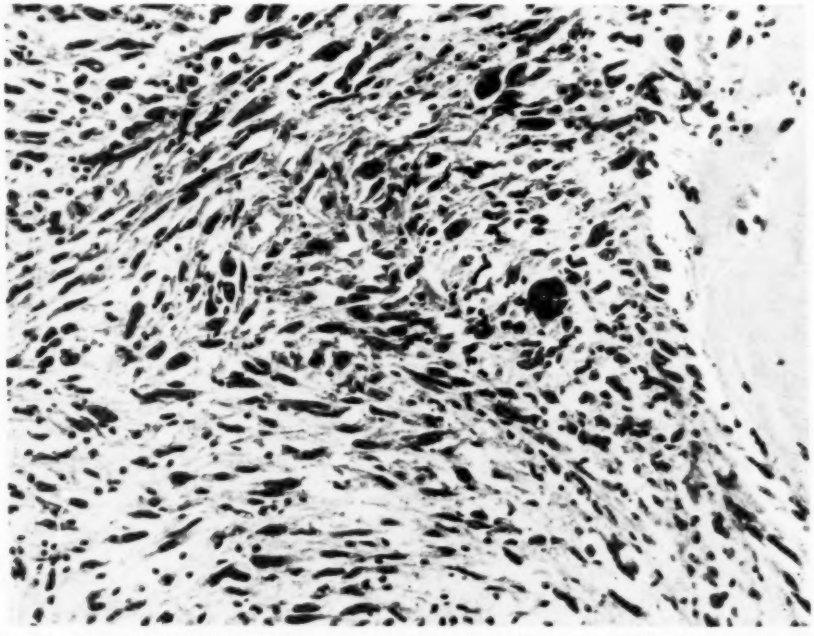

FIGURE 5.—Spleen (A 1748) of a C_3H mouse. The margin of the space occupied by the methylcholanthrene pellet is at the right in the illustration. Bordering the pellet space and extending into the splenic tissue is an area of atypical spindle cells showing a tendency to be arranged in interlacing bundles. Photomicrograph $\times 285$.

Of the 20 mice, the liver contained a hepatoma in 2 and an encysted worm in a third, while enlarged axillary and inguinal nodes were present in another.

INTRAHEPATIC INJECTION AND IMPLANTATION

EXPERIMENT 1A

A cotton thread coated with dibenzanthracene was stitched (November 30, 1938) into the livers of eight young male mice of the C57 Black strain. The animals survived for the following periods: 9, 11, 12, 12, 13, 13, 14, and 16 months. One animal developed an adenocarcinoma of the liver with peritoneal implantation. In the livers of three other animals the threads were encapsulated with hyalinized connective tissue around which there was a foreign body reaction. In one of these there was a small focus of hyperplastic bile ducts adjacent to the encapsulated thread.

The animal which developed the adenocarcinoma of the liver died 12 months after insertion of the thread. The peritoneal cavity was distended with blood-stained fluid. The anterior surface of the liver was adherent to the abdominal wall. On the under surface of the liver, there was a large, firm growth, 15×25 mm., mottled gray and red. The lesion was firm throughout, and portions of the tissue were grayish white. On the surface of the right kidney were several small white areas, the largest of which, at the pelvis of the left kidney, measured 4×4×6 mm. The visceral and parietal peritoneum contained small gray nodules on the surface.

Microscopic examination of this lesion showed an adenocarcinoma directly infiltrating the liver tissue (fig. 6). The tumor was composed of glands and nests of tumor cells. The acini varied in shape and in size from small spaces to large cystic

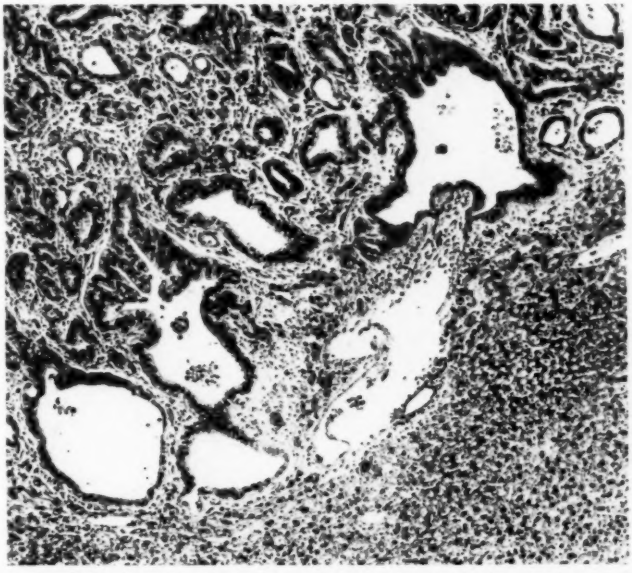

FIGURE 6.—Liver (A5874) of a C57 Black mouse showing an area of adenocarcinoma infiltrating liver tissue. Photomicrograph ×80.

structures; some were imperfectly formed. Most of the tumor cells were columnar, but some were cuboidal and others flattened. The nuclei varied in size, some being exceedingly large. Only one or two mitotic figures were observed in several high-power fields. The stroma, which was not abundant, consisted of mature connective tissue. The blood vessels were few in number and well-formed. The tumor tissue showed extensive necrosis. The gall bladder was not identified in any of the sections. The secondary deposits in the peritoneum, diaphragm, and kidney resembled the primary tumor.

Experiment 1B

A cotton thread coated with benzpyrene was stitched (November 30, 1938) into the livers of seven young C57 Black mice (six females and one male). The animals survived for the following periods: 1, 7, 7, 9, 9, 10, and 10 months. No significant change was found in any of the animals. The encapsulated thread, with the previously described foreign-body reaction about it, was seen in two cases microscopically.

Experiment 1C

A cotton thread coated with methylcholanthrene was stitched (November 30, 1938) into the livers of one female and five male mice of the C57 Black strain. Two animals survived for 10 and 11 months, and the other four mice for 12 months. The encapsulated thread was seen microscopically in the liver in one mouse. Another animal showed focal areas of myeloid cell infiltration in the liver. The liver of the third animal contained an area of multiple cysts which varied considerably in size. The lining cells were thin and flattened, and were difficult to discern in some cysts. The lumen of the spaces contained finely granular acidophilic material.

Experiment 2

Wool yarn was soaked in a lard solution containing 4 mg. methylcholanthrene per cubic centimeter and then sutured (May 4, 1937) through the liver and tied. Eleven C_3H female mice were used in this experiment. As a control, yarn soaked in lard was similarly sutured through the liver in 10 other C_3H females. Three of the test mice died after 4, 6, and 7 months, respectively; the other 8 animals survived for 9, 11, 12, 12, 13, 16, 16, and 18 months. The encapsulated yarn was seen, microscopically, in the livers of 5 of the animals which died between 4 and 16 months.

In one case the encapsulated yarn was present on the surface of the liver adjacent to the diaphragm. There was a foreign-body reaction about it and, in addition, a marked proliferation of atypical spindle cells in the capsule of the liver. At some distance from the lesion was a small hepatoma. In another mouse the fragment of yarn in the liver was surrounded by a capsule of connective tissue containing calcific deposits; the liver of this mouse also contained a small cyst and an infiltration of myeloid cells.

No significant changes were noted in the livers of the mice of the control group.

Experiment 3

From 0.03 to 0.05 cc. of a solution containing 10 mg. methylcholanthrene per cubic centimeter of light mineral oil was injected (September 29, 1937) into the livers of six strain A mice (one female and five males). The mice survived for 1, 5, 7, 9, 11, and 15 months.

In the mouse that died after 1 month, the liver contained a fibrous, encapsulated space which presumably represented the site of injection. In the mouse dead after 11 months there was a slight increase in fibrous tissue around the portal areas of the liver.

Intra-abdominal spindle-cell sarcomas developed in two mice after 5 and 9 months, respectively. In each case there was a small ulcer of the upper anterior abdominal wall beneath which was a tumor measuring 15 and 22 mm. in diameter, respectively. Both tumors were adherent to the lower border of the liver in one small area.

EXPERIMENT 4

Methylcholanthrene pellets (*12*) were implanted (April 22, 1937) into the livers of 15 young male C_3H mice. The pellets were seen intact at autopsy in 13 of the 15 mice which survived as follows: 2 mice for 5 and 6 months, 9 mice between 9 and 12 months, and 4 mice for 16 months.

On microscopic examination of sections of the liver, the pellet space was surrounded by an area of hyalinized connective tissue in all cases (fig. 7). In a few livers, there was a slight infiltration of pigmented and vacuolated foreign-body giant cells. In another, there was a localized area of cirrhosis and bile duct hyperplasia near the pellet. Hepatomas developed in three animals. In two they occurred at a site distant from the pellet, and in the third the pellet was found imbedded in the hepatoma. These hepatomas corresponded in gross and microscopic appearance with those which occur spontaneously in a high proportion of C_3H mice.

FIGURE 7.—Liver (A 826) of a C_3H mouse. The space occupied by the methylcholanthrene pellet lies near the surface of the liver and is surrounded by an area of hyalinized connective tissue. The hepatic tissue in contact with this capsule shows no change. Photomicrograph × 48.

DISCUSSION

Intrasplenic implantation of pellets of methylcholanthrene was successful in producing sarcoma in 5 of the 12 mice that lived for more than 7 months. In 2 others there was an area of proliferation of atypical spindle cells adjacent to the pellet; these lesions were observed at a stage which was not sufficiently advanced to justify a diagnosis of malignancy. While in this experiment tumors of the spleen were induced in a higher proportion of the animals than in analogous experiments reported by others, the response of the spleen to methylcholanthrene nevertheless was not so pronounced as that of many other tissues. From this experiment, in conjunction with other reports in the literature, it would appear that the spleen is less susceptible to the action of carcinogenic hydrocarbons than the subcutaneous tissues but more susceptible than the liver.

The rather meager data in the literature reveal two instances of tumors of the liver, both sarcomas, induced after 16 and 25 months in rats by implantation of hydrocarbon carcinogens directly into liver tissue. In mice, the two liver tumors reported by Ilfeld (*1*) are frequently cited as tumors induced by this technique. It is pointed out, however, that these tumors were obtained in C_3H mice, a strain which has a high incidence of spontaneous liver tumors.

This fact was not appreciated at the time of Ilfeld's work. The histologic structure of one of these tumors was obscured by post-mortem change. Judging from Ilfeld's description and illustration of the other hepatic tumor, this growth was similar to the spontaneous hepatomas which occur so frequently in this strain of mouse. The possibility, therefore, exists that these two tumors may well have been spontaneous in origin.

Similarly the 4 hepatomas obtained in our series of 53 mice all occurred in animals of the C_3H strain. The incidence of these 4 tumors in 26 C_3H mice is well within the incidence of spontaneous hepatomas in this strain in our experience and in that of others (*14, 15*). Moreover, their gross and microscopic appearance was similar to that of the spontaneous hepatoma of this strain.

Mice of the C_3H strain were deliberately included for this type of experiment because of their tendency to develop spontaneous hepatic tumors. It has been found (*16*) less difficult to induce pulmonary tumors with hydrocarbon carcinogens in mice of strains which have a pronounced tendency to develop pulmonary tumors spontaneously than in mice which have a low tendency. In the susceptible mice the induced pulmonary tumors appear much earlier, in a higher percentage of the mice, and in greater numbers in each mouse, than do the spontaneous pulmonary tumors. It was therefore anticipated that mice with a marked tendency to develop hepatic tumors spontaneously might be particularly susceptible to the carcinogenic action of hydrocarbons implanted in the liver. This, however, did not prove to be the case in these experiments.

Two intra-abdominal sarcomas were obtained in intrahepatic experiment 3, in which mineral oil was the vehicle. These spindle-cell sarcomas were situated in the deeper aspect of the anterior abdominal wall, projected into the abdominal cavity, and were adherent to the liver. They did not invade the liver deeply and did not appear to be primary sarcomas of this organ; they rather seemed to have originated in the anterior abdominal wall and to have become adherent to the liver secondarily. Some of the injected mineral oil was sometimes seen to have leaked out through the needle tract as the needle was withdrawn from the liver and thus accidentally came in contact with the peritoneum and with the incised tissues of the abdominal wall. This could readily have accounted for the development of these two sarcomas.

In one mouse there was marked proliferation of atypical spindle cells around the impregnated yarn lying on the surface of the liver near the diaphragm. From the position of the lesion it was considered that these spindle cells were probably derived from the liver capsule or from the diaphragm, rather than from hepatic tissue.

It is uncertain whether the solitary and multiple cysts, the bile-duct proliferation in several animals, and the adenocarcinoma of the liver were spontaneous or induced. However, in view of the position of these lesions in relation to the implanted carcinogens, it is possible that they may have been induced. The adenocarcinoma was an unusual tumor. From its structure it did not appear to be of liver-cell derivation, but may have originated from the lining cells of the biliary system, either of the bile ducts or of the gall bladder. Its position on the under surface of the liver, involving the hilum, where the gall bladder and large bile ducts are located, lends further support to this interpretation. Attesting to its high degree of malignancy were its widespread infiltration of liver tissue and multiple metastases.

Even if the foregoing lesions were in-

duced, the experiments in this laboratory indicate a high resistance of the liver in mice and rats to the carcinogenic action of implanted hydrocarbons. Neither in the 110 rats of the unpublished experiments of Jones, Rothman, and Shear, nor in the 53 mice described in this paper, was there an unequivocal production of hepatic tumors. In addition to the above-described lesions, proliferation of atypical spindle cells in the region of the carcinogen occurred in varying degree in a few of the mice and rats, but in no case did this progress to the formation of a malignant tumor. It is of interest that the only 2 tumors reported (7) as having been definitely induced in the liver (rat) by implanted carcinogen required 16 and 25 months, respectively.

The negligible response of the liver to these carcinogens is obviously not to be ascribed to absorption or destruction of the hydrocarbons. The pellets of methylcholanthrene which were recovered intact in 13 of 15 mice had been present, in some animals, for periods as long as 16 months. When similar pellets were placed in the subcutaneous tissues and in the brain of mice of the same strain, tumors were obtained (12) in a high proportion of the animals in a much shorter time.[8] The unexpectedly mild tissue reaction around the methylcholanthrene pellets in the liver therefore contrasts curiously with the greater response of other tissues similarly treated.

This resistance to the carcinogenic action of implanted hydrocarbons is not to be ascribed to any special refractivity on the part of the liver to induction of tumors inasmuch as tumors of the liver have been readily produced by condensed polycyclic compounds containing ring nitrogen (17), by 2-amino-5-azotoluene (18),[9] and by dimethyl-amino-azobenzene (19). On the contrary, the fact that hepatic tumors have been produced readily in experiments in which the compounds were not injected directly into the liver, but were painted, fed, or injected subcutaneously, demonstrates that the liver is a susceptible tissue provided compounds of suitable specificity for the liver are employed. This again emphasizes the point made previously in these studies (20), as well as by others, that carcinogenicity is not a property inherent in a compound considered independently of the details of the biological experiments, but rather that it depends upon a variety of factors including the tissue under consideration.

SUMMARY

1. Intrasplenic implantation of pellets of methylcholanthrene was made in 20 C_3H mice. Sarcomas of the spleen were obtained in 5 of the 12 mice that lived for more than 7 months.

2. Three carcinogenic hydrocarbons (1, 2,5,6-dibenzanthracene, 3,4-benzpyrene, and 20-methylcholanthrene) were introduced, by various techniques, directly into the liver of 53 mice of several strains. Relevant lesions occurred in 7 (4 hepatomas, 1 solitary cyst, 1 case of multiple

[8] *Stewart (unpublished experiments, 1940) has produced sarcomas of the testicle in a comparable time with methylcholanthrene. Carcinogenic hydrocarbons were introduced directly into the testicle in 40 C_3H mice. A lard solution of methylcholanthrene (7 mice), threads soaked in a lard solution of methylcholanthrene (12 mice), and threads coated with solid dibenzanthracene (6 mice) or solid benzpyrene (6 mice), failed to produce tumors in experiments lasting for periods between 12 and 20 months; atrophy, fibrosis, foreign body reaction, and calcification of the testicle were noted in many of the animals. However, threads coated with solid methylcholanthrene produced sarcomas involving the testicle in 6 of 9 mice in from 6 to 11 months.*

The different results obtained with the different procedures of introducing methylcholanthrene again illustrate the importance of the technique. With the same technique, of threads coated with solid hydrocarbon, the discrepancy between the positive results obtained with methylcholanthrene and the negative results with dibenzanthracene and with benzpyrene was, however, unexpected.

[9] *This paper gives the bibliography of the work of Yoshida with this compound.*

cysts, and 1 adenocarcinoma); the spontaneous or induced origin of these lesions is discussed.

3. The low degree of response of the liver is not due to absorption and removal of hydrocarbon, for the implanted methylcholanthrene pellets were recovered intact in the livers in 13 of 15 mice.

4. The failure to obtain unequivocally induced tumors of the liver in a considerable proportion of the mice indicates that the liver is refractory to the carcinogenic action of these hydrocarbons. The spleen appears to have a susceptibility less than that of the subcutaneous tissues but greater than that of the liver.

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Studies in Carcinogenesis

XIV. 3-Substituted and 10-Substituted Derivatives of 1,2-Benzanthracene [1] [2]

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Cook and Kennaway (*3*) have recently stated: "In the study of carcinogenic properties of new chemical compounds it is almost inevitable that considerable time should elapse between publication of the synthesis of a compound and of the final report of its biological testing." The present paper records the results obtained on injecting, subcutaneously into mice, 66 derivatives of 1,2-benzanthracene.

Earlier experiments from this laboratory (*4*) had demonstrated the importance, for carcinogenic potency, of methyl substitution at the 10-position of 1,2-benzanthracene, and had shown (*5*) that substitution at the 3-position conferred carcinogenic potency in some instances. Consequently a considerable number of compounds with substituents at these positions were synthesized [3] and examined for tumor-producing potency [4] in mice.

For the most part the compounds were injected as in previous experiments, i. e., in the crystalline form moistened with glycerol. Unless otherwise specified, only one injection was given, subcutaneously, in the left flank of strain A mice, which were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The properties of the specimens employed have been described in the papers from the cooperating laboratories of organic chemistry. Solutions in lard filtered at 38° C. were used in those instances in which not enough of the compound was available for administration of the required amount in crystalline form, or when low doses were desired.

The animals were fed a diet of Purina dog chow and tap water, as in previous experiments in this series.

In the tables the plus signs give the total number of mice, in each experiment, in which tumors had been induced in the times shown; the minus signs give the number of negative survivors at those times. In the tables and text the term "negative" designates the mice not bearing induced tumors at the site of application.

10-SUBSTITUTED DERIVATIVES

AROMATIC HYDROCARBONS

A series of seven closely related, completely aromatic hydrocarbons was examined for potency. This series consisted of 1,2-benzanthracene derivatives with the

[1] *This work was carried out in the United States Public Health Service, Office of Cancer Investigations, then located at the Wolcott Gibbs Memorial Laboratory, Harvard University.*

[2] *Presented (1) at the 16th International Physiological Congress, Zurich, Switzerland, August 14, 1938. A later summary was included in the seventh paper (2) of this series.*

[3] *The majority of these compounds were synthesized in the laboratory of Prof. L. F. Fieser of Harvard University as part of a cooperative project initiated by the United States Public Health Service in 1934. A considerable number were synthesized in the laboratory of Doctor M. S. Newman of the Ohio State University. Unless otherwise designated, the compounds were those of Professor Fieser and coworkers.*

[4] *The results obtained in these biological experiments were announced in the papers of Fieser and coworkers and of Newman and coworkers in the Journal of the American Chemical Society, which described the synthesis of these compounds, and in the reviews by Fieser and others (5) and by Fieser (6).*

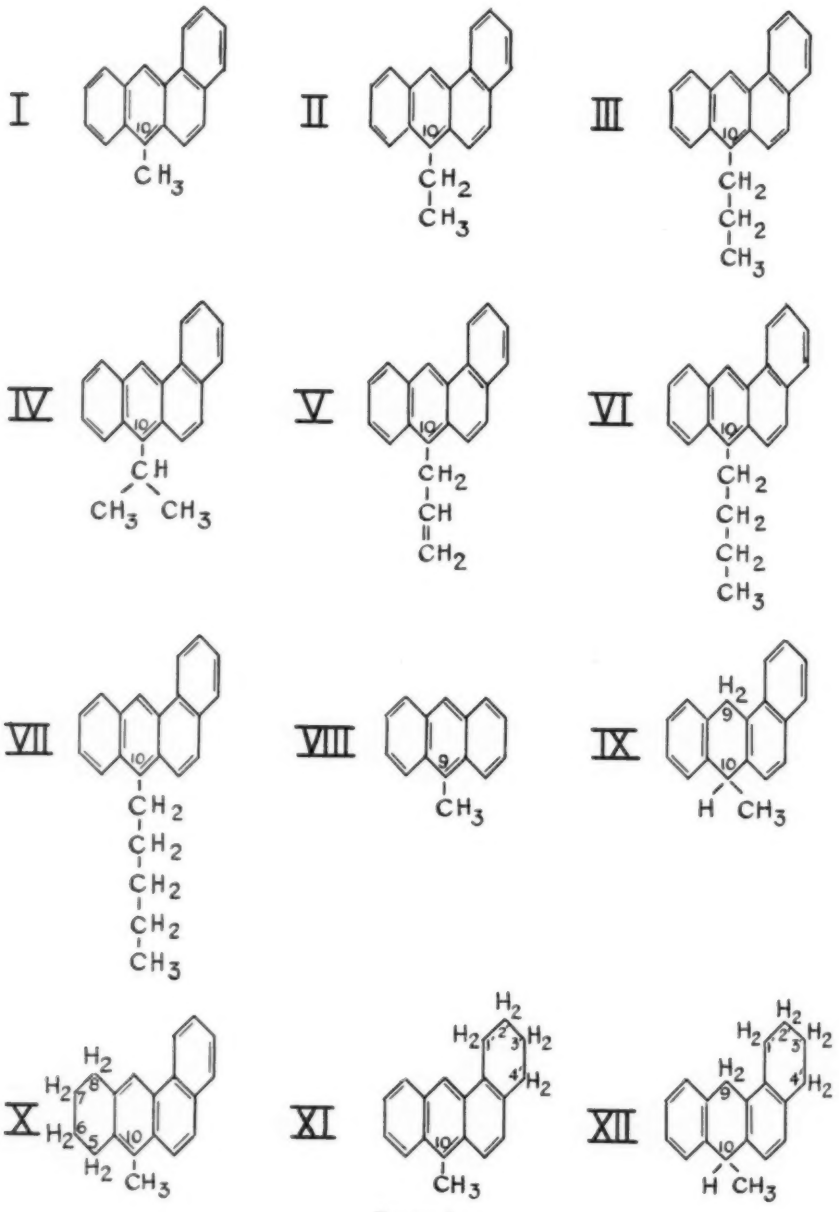

FIGURE 1.

following substituents: 10-CH_3; 10-$CH_2{\cdot}CH_3$; 10-$CH_2{\cdot}CH_2{\cdot}CH_3$; 10-$CH{\cdot}(CH_3)_2$; 10-$CH_2{\cdot}CH{:}CH_2$; 10-$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_3$; and 10-$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_3$. For comparison, the 9-$CH_3$ derivative of anthracene was also examined.

10-Methyl-1,2-benzanthracene (fig. 1, formula 1).—The results obtained following the subcutaneous injection of 5 mg. of crystalline 10-methyl-1,2-benzanthracene into strain A mice, and of 1 mg. in filtered lard into C_3H mice, as well as those obtained following skin painting of strain A mice with a 0.3-percent solution in benzene, have been described in a previous report (*4*).

Because of the pronounced skin damage and general toxic effects produced by painting with a 0.3-percent solution, two lower concentrations were subsequently employed. Twenty strain A mice (10 males and 10 females) were painted twice weekly with a 0.1-percent solution in benzene for 7 months (November 24, 1937–June 17, 1938), when the painting was discontinued; a duplicate experiment was carried out at the same time with a 0.02-percent solution in benzene. The results are summarized in table 1 which also gives for comparison the data previously obtained with the 0.3-percent solution.

TABLE 1.—*10-Methyl-1,2-benzanthracene*

[Production of cutaneous tumors in strain A mice following skin painting]

Concentration of hydrocarbon in benzene	Mice		Total number of mice with tumors (+), surviving mice with papillomas (p), and negative surviving mice (−), after various periods (in months)			
	Number	Sex	6 months	9 months	12 months	16 months
Percent						
0.3	20	M+F	4+ 3p 5−	11+ 0p 0−	.....	.....
.1	20	M+F	2+ 0p 13−	2+ 2p 9−	4+ 1p 6−	4+ 1p 1−
.02	20	M+F	0+ 0p 20−	0+ 0p 12−	0+ 0p 9−	0+ 1p 6−

TABLE 2.—*10-Methyl-1,2-benzanthracene*

[Production of subcutaneous tumors in mice]

Dose and medium	Mice			Total number of mice with tumors (+), negative surviving mice (−), after various periods (in months)		
	Number	Sex	Strain	4 months	6 months	9 months
Mg.						
5 (as crystals)	30	M	A	23+ 6−	25+ 1−	26+ 0−
1 (in filtered lard)	20	M+F	C_3H	9+ 9−	16+ 1−	.....
0.05 (in filtered lard)	20	M+F	A	5+ 15−	6+ 14−	7+ 9−

The skin damage, toxic effects, and high early mortality noted in the experiment with the 0.3-percent solution were less pronounced with the 0.1-percent, and still less so with the 0.02-percent solution. The reduction of the local and general ill effects and the greater longevity did not, however, result in an increase in the number of induced tumors.

For studying the action of promoting factors in the genesis of skin tumors with this hydrocarbon, 0.1 percent appears to be a more suitable concentration than 0.3 percent. For similar studies on subcutaneous tissues, Sall and Shear (*7*) found 0.05 mg. of this carcinogen to be preferable to either 5 mg. or 1 mg. (*4*). These data are brought together in table 2.

It is now clear that the apparent divergence between the results obtained in London (*8, 9*) and in this laboratory with 10-methyl-1,2-benzanthracene was a consequence of the use of different sites of application. When we first announced that 10-methyl-1,2-benzanthracene was quite potent, and that substitution at the meso-position 10 seemed to be particularly im-

portant, the London workers considered this conclusion as "premature," apparently because they had obtained, with the skin-painting technique, evidence of only weak potency. We had obtained, by the subcutaneous route, pronounced potency. Consequently we repeated the subcutaneous experiment and also applied the 10-methyl derivative to the skin of other mice of the same strain. Confirmation of the high potency of this compound for the subcutaneous tissues and of its lower potency for the skin was obtained. This illustrates again the necessity of considering conclusions regarding carcinogenic potency not as an invariable property of the compound alone but also, among other factors, of the tissue tested.

10-Ethyl-1,2-benzanthracene (fig. 1, formula II).—It was reported in a previous paper (*4*) that the significance of the 10-position was further attested by the potency of the 10-ethyl derivative. This hydrocarbon produced tumors in a high proportion of the mice, although the latent period was longer than in the case of the 10-methyl compound.

Five milligrams of this hydrocarbon was injected (December 12, 1936) into 20 strain A male mice. As the mice were still negative after 3 months, 10 mg. more were injected in each mouse at this time. Ulceration at the site of injection was noted in 5 mice after 5 months, and in several others subsequently.

The first tumor appeared in 4 months; the hydrocarbon was seen at autopsy imbedded in the tumor. Tumors developed in a total of 16 mice in the course of a year (table 3). This compound is, therefore, a highly potent carcinogen, but it acts more slowly than its 10-methyl analogue.

10-Propyl-1,2-benzanthracene (fig. 1, formula III).—The 10-propyl derivative was injected (March 16, 1937) into 10 strain A and 10 C_3H male mice. Since no tumors had appeared, the injection of 5 mg. was repeated after 4 months and again after

TABLE 3.—*10-Substituted aromatic hydrocarbon derivatives of 1,2-benzanthracene*

[Production of subcutaneous tumors in mice]

Substituent	Formula No.	Dose (crystals)	Mice			Total number of mice with tumors (+), and negative surviving mice (−), after various periods (in months)				
			Number	Sex	Strain	4 months	6 months	9 months	12 months	15 months
		Milligrams								
10-CH_3 (methyl)	I	5	30	M	A	23+ 6−	25+ 1−	26+ 0−		
10-C_2H_5 (ethyl)	II	15	20	M	A	1+ 19−	7+ 13−	13+ 6−	16+ 2−	
10-C_3H_7 (propyl)	III	15	20	M	A+C_3H	0+ 20−	0+ 20−	0+ 20−	0+ 17−	0+ 10−
10-C_3H_7 (*iso*-propyl)	IV	20	20	M	A+C_3H	0+ 20−	0+ 19−	0+ 18−	0+ 17−	0+ 11−
10-C_3H_5 (allyl)	V	15	20	M	A+C_3H	0+ 20−	0+ 19−	0+ 16−	0+ 7−	
10-C_4H_9 (butyl)	VI	20	20	M	A+C_3H	0+ 20−	0+ 20−	0+ 20−	0+ 18−	0+ 11−
10-C_5H_{11} (amyl)	VII	15	20	M+F	A	0+ 20−	0+ 19−	0+ 16−	0+ 14−	
9-methylanthracene [1]	VIII	7	20	M+F	A	0+ 20−	0+ 20−	0+ 18−	0+ 9−	0+ 4−

[1] This anthracene derivative was examined for comparison with 10-methyl-1,2-benzanthracene. Compare fig. 1, formulas I and VIII.

9 months. No tumors were obtained in this experiment, which lasted for 15 months, by the time the 10 surviving mice were killed. The compound was seen at the injection site in all 10 mice at autopsy 6 months after the last injection. The data are summarized in table 3.

*10-*iso*-Propyl-1,2-benzanthracene* (*fig. 1, formula IV*).—Five milligrams of the 10-isopropyl compound were injected (March 16 1937) into 10 strain A and 10 C_3H male mice. The same dose was repeated after 4, 9, and 13 months.

The experiment was terminated after 15 months at which time there were 11 survivors. No tumors were obtained at the site of injection in any of the mice. This result is in agreement with the negative finding in a skin-painting experiment with this hydrocarbon in London (*10*). The data are summarized in table 3.

10-Allyl-1,2-benzanthracene (*fig. 1, formula V*).—The 10-allyl compound was injected (April 17, 1937) into 10 strain A and 10 C_3H male mice. The injection of 5 mg. was repeated after 3 and after 8 months. No tumors were obtained at the injection site in any of the mice.

The hydrocarbon was seen in the subcutaneous tissues at autopsy in many of the mice, including the last survivor killed at the end of 13 months; i. e., 5 months after the last injection. The data are summarized in table 3.

10-Butyl-1,2-benzanthracene (*fig. 1, formula VI*).—The 10-butyl compound was also injected (March 3, 1937) into 10 strain A and 10 C_3H male mice. The dose of 5 mg. was repeated after 4, 9, and 13 months. No tumors were obtained at the injection site in any of the mice. The experiment was terminated after 15 months when the 11 survivors were killed. The data are summarized in table 3.

10-Amyl-1,2-benzanthracene (*fig. 1, formula VII*).—Twenty strain A mice, half males and half females, were given (May 11, 1937) 5 mg. of the 10-amyl derivative. The injection was repeated after 2 and after 9 months. No tumors at the injection site were obtained in any of the mice in 14 months, when the 11 survivors were killed. At autopsy, 5 months after the last injection, the hydrocarbon was seen at the site of injection in 10 of these 11 mice. The data are summarized in table 3.

9-Methylanthracene (*fig. 1, formula VIII*).—For comparison with 10-methyl-1,2-benzanthracene, the analogous methylanthracene [5] was injected into 20 strain A mice of both sexes. Two milligrams of the hydrocarbon was injected (October 17, 1938) initially, and 5 mg. was given at the end of 6 months. No tumors developed either in the 4 survivors that were killed after 15 months, or in any of the mice that died during the course of the experiment (table 3). Velluz (*11*) has reported negative results in a skin-painting experiment in mice with this compound.

HYDROGENATED HYDROCARBONS

Four derivatives of 10-methyl-1,2-benzanthracene, which were hydrogenated in varying degrees, were examined. These four compounds all gave negative results.

9,10-Dihydro-10-methyl-1,2-benzanthracene (*fig. 1, formula IX*).—Five milligrams of the 9,10-dihydro-10-methyl derivative was injected (July 20, 1937) into 10 strain A male mice, and the injection was repeated after 4 and after 9 months. No tumors were obtained in this experiment, which lasted for 14 months (table 4).

5,6,7,8-Tetrahydro-10 methyl-1,2-benzanthracene (*fig. 1, formula X*).—Two injections, 3 months apart, of 5 mg. each of the 5,6,7,8-tetrahydro-10-methyl derivative were given (July 20, 1937) to 10 strain A

[5] *Compare formulas I and VIII in fig. 1.*

TABLE 4.—*Hydrogenated derivatives of 10-methyl-1,2-benzanthracene*

[Results of subcutaneous injection in mice]

Positions hydrogenated	Formula No.	Dose (crystals)	Mice			Total number of mice with tumors (+), and negative surviving mice (−), after various periods (in months)			
			Number	Sex	Strain	6 months	9 months	12 months	15 months
		Milligrams							
9,10-dihydro	IX	15	10	M	A	0+ 10−	0+ 9−	0+ 2−	.....
5,6,7,8-tetrahydro	X	10	20	M	A+C_3H	0+ 19−	0+ 15−	0+ 12−	0+ 6−
1′,2′,3′,4′-tetrahydro	XI	10	10	M	A	0+ 10−	0+ 6−	0+ 3−	0+ 1−
1′,2′,3′,4′,9,10-hexahydro	XII	[1] 20	20	M+F	A	0+ 19−	0+ 18−	0+ 8−	0+ 3−

[1] In lard filtered at 38° C.

and 10 C_3H male mice. At the end of 15 months the 6 survivors were killed. No tumors had developed in any of these mice at the injection site (table 4).

1′,2′,3′,4′-Tetrahydro-10-methyl-1,2-benzanthracene (*fig. 1, formula XI*).—Five milligrams of the 1′,2′,3′,4′-tetrahydro-10-methyl derivative was injected (June 4, 1937) into 10 strain A male mice. The injection was repeated after 5 months. During the seventh month ulceration at the site of injection developed in 3 mice. However, no tumors had developed by the time the last survivor was killed in the sixteenth month (table 4).

1′,2′,3′,4′,9,10-Hexahydro-10-methyl-1,2-benzanthracene (*fig. 1, formula XII*).—The 1′,2′,3′,4′,9,10-hexahydro-10-methyl derivative, a pale yellow liquid, was dissolved in lard filtered at 38° C. to give a solution containing 50 mg. of the hydrocarbon per cubic centimeter. One-tenth cubic centimeter of this solution, equivalent to 5 mg. of the hydrocarbon, was injected (November 8, 1937) into 10 male and 10 female mice of the A strain. The injection was repeated after 4, 5, and 12 months. No tumors had appeared at the site of injection by the time the last 3 survivors were killed in the sixteenth month (table 4).

OXYGEN-CONTAINING DERIVATIVES

Ten oxygen-containing derivatives related to 10-methyl-1,2-benzanthracene, in which the oxygen-containing groups were in the 10-position, were examined. The fact that several of these were carcinogenic is regarded as having considerable interest.

An interesting series of 10 compounds was obtained through Professor Fieser from Prof. J. von Braun, formerly of the Chemical Institute of the University of Frankfurt a. M. All of these compounds [6] were derivatives of 10-methyl-1,2-benzanthracene with the substituents at the 10-methyl carbon; in 5 of them the substituents contained oxygen.

10-Hydroxymethyl-1,2-benzanthracene (*fig. 2, formula XIII*).—10-Hydroxymethyl-1,2-benzanthracene (m. p. 174°–176° C.) was injected (August 16, 1937) into 10 strain A male mice in a dose of 5 mg. which was repeated after 5 months. The first tumor appeared in the eighth month. The 4 tumors that developed in these mice all appeared during the first 13 months. The experiment was terminated in the nineteenth month (table 5). In 4 of the mice there were multiple tumors of the

[6] *These compounds were stated to have been synthesized by J. von Braun and E. Kamp. The syntheses have not been published except for the material which appeared in Kamp's Dissertation* (*12*).

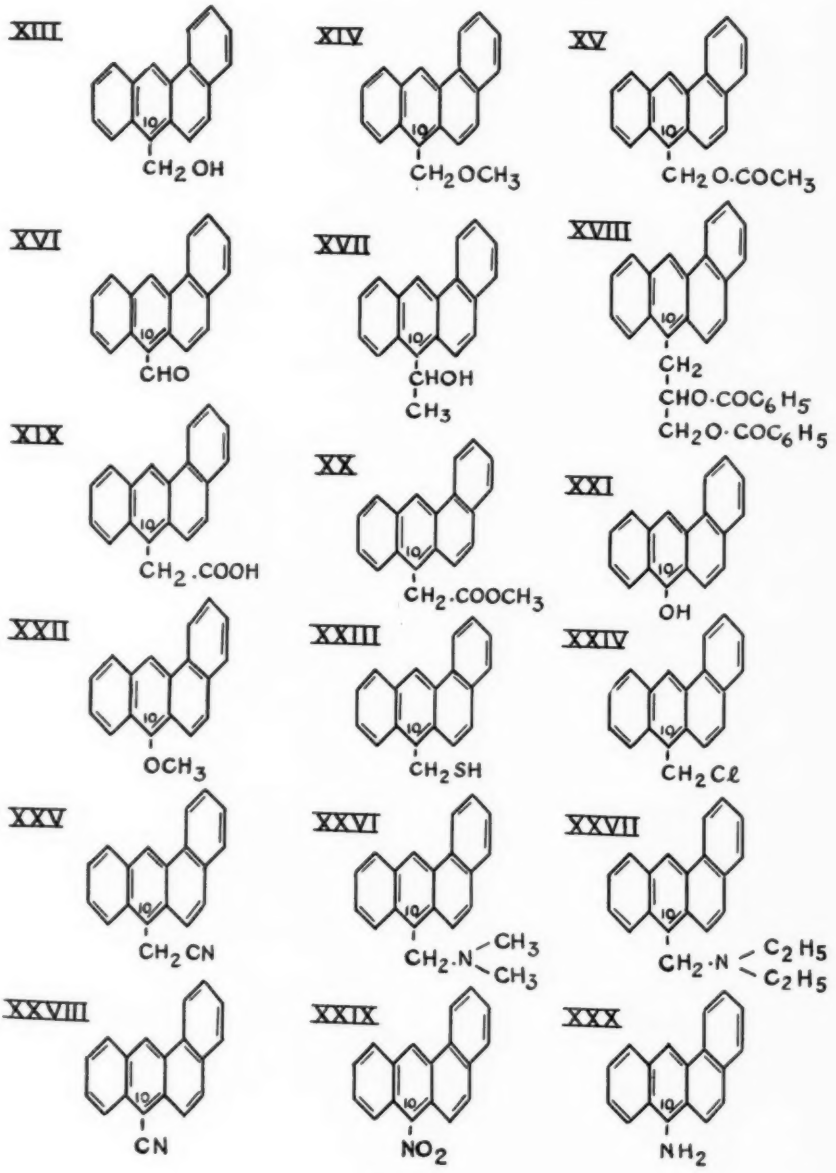

FIGURE 2.

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lungs; in 2 of these cases the tumors were very numerous.

Cook and Kennaway (*3*) recently stated that malignant tumors in mice have also been obtained in their laboratory with this compound. In their confirmatory experiment, sarcomas were obtained in all of the 10 mice employed. In addition, eptheliomas were obtained in 3 of 10 mice painted with an acetone solution of this compound.

10-Methoxymethyl-1,2-benzanthracene (*fig. 2, formula XIV*).—No tumors were obtained with the 10-methoxymethyl derivative (m. p. 124° C.) of Von Braun, although ulceration occurred at the injection site in 2 mice in the seventh month. Two injections of 5 mg. each were given (August 18, 1937, and January 12, 1938) to 10 strain A male mice. The experiment lasted for 18 months (table 5).

10-Acetoxymethyl-1,2-benzanthracene (*fig. 2, formula XV*).—The most potent of the 10 compounds of Von Braun was the 10-acetoxymethyl derivative (m. p. 149–150° C.). The dose injected (August 18, 1937) of 5 mg., was repeated after 5 and after 8 months. Ten strain A male mice were employed.

The first two tumors developed in the sixth month at the margin of the ulcers which occurred in two mice. Tumors developed in 3 others by the end of a year. The last survivor developed a tumor in the fifteenth month. This compound induced tumors in 8 of the 10 mice and is therefore a highly potent carcinogen, although slowly acting (table 5).

Badger and Cook (*13*) mentioned recently that Kennaway has also obtained malignant tumors in mice with this compound. The details of the confirmation of the potency of this compound have not as yet been published.

TABLE 5.—*Oxygen-containing derivatives of 10-methyl-1,2-benzanthracene*

[Production of subcutaneous tumors in mice]

Substituent	Formula No.—	Dose (crystals)	Mice			Total number of mice with tumors (+), and negative surviving mice (−) after various periods (in months)			
			Number	Sex	Strain	6 months	9 months	12 months	15 months
10-CH_2OH	XIII	*Milligrams* 10	10	M	A	0+ 10−	1+ 9−	3+ 7−	4+ 5−
10-CH_2OCH_3	XIV	10	10	M	A	0+ 9−	0+ 8−	0+ 6−	0+ 4−
10-CH_2O-$COCH_3$	XV	15	10	M	A	2+ 8−	2+ 6−	5+ 3−	8+ 0−
10-CHO	XVI	7	20	M+F	A	0+ 19−	7+ 6−	10+ 0−	.....
10-CHOH-CH_3	XVII	7	20	M+F	A	0+ 19−	0+ 16−	0+ 10−	0+ 7−
10-Propylene glycol dibenzoate.	XVIII	15	10	M	A	0+ 10−	0+ 9−	0+ 9−	0+ 7−
10-CH_2-COOH	XIX	15	10	M	A	0+ 9−	0+ 9−	0+ 7−	0+ 2−
10-CH_2-$COOCH_3$	XX	15	10	M	A	0+ 10−	3+ 6−	4+ 5−	5+ 2−
10-OH	XXI	25	20	M+F	C57 Brown	0+ 18−	0+ 16−	0+ 10−	.....
10-OCH_3	XXII	15	20	M	A+C_3H	1+ 19−	3+ 17−	5+ 15−	8+ 9−

1,2-Benzanthracene-10-aldehyde (*fig. 2, formula XVI*).—The 10-aldehyde of 1,2-benzanthracene, synthesized in Professor Fieser's laboratory by Dr. J. L. Hartwell, of the National Cancer Institute, was injected (October 17, 1938) into 10 male and 10 female mice of the A strain in a dose of 2 mg. An injection of 5 mg. was given after 5 months.

The first tumor appeared in the seventh month. During the next 3 months tumors appeared in 9 more mice, making a total of 10 induced tumors in 10 months. The two survivors that were negative at this time died in the eleventh month without evidence of tumor (table 5).

α-Hydroxy-10-ethyl-1,2-benzanthracene (*fig. 2, formula XVII*).—This compound, synthesized by Dr. Hartwell in Professor Fiesers's laboratory, was of interest in that it contained an asymmetric carbon atom. It gave, however, no tumors.

Two milligrams was injected (October 17, 1938) into 10 male and 10 female mice of the A strain. An injection of 5 mg. was given 6 months later. During the seventh month ulceration occurred at the injection site in 2 mice, but no tumors developed in any of the animals at the site of injection although the experiment lasted 15 months (table 5).

3-(1,2-Benz-10-anthranyl)-1,2-propyleneglycol dibenzoate (*fig. 2, formula XVIII*).—The 10-propyleneglycol dibenzoate of 1,2-benzanthracene was injected (May 11, 1937) into 10 strain A male mice in a dose of 5 mg. which was repeated after 2 and after 9 months. No subcutaneous tumors were obtained although the compound was found at autopsy at the injection site in the mice that died 8 months after the last injection. This experiment lasted 17 months (table 5).

10-Carboxymethyl-1,2-benzanthracene (*fig. 2, formula XIX*).—Five milligrams of the 10-carboxymethyl derivative (m. p. 281°–282° C.), one of the compounds of Von Braun, was injected (August 20, 1937) into 10 strain A male mice. The injection was repeated after 5 and after 8 months. No subcutaneous tumors were obtained in this experiment which was terminated after 15 months (table 5).

10-Carbomethyoxymethyl-1,2-benzanthracene (*fig. 2, formula XX*).—Although the 10-carboxymethyl derivative was inactive, the methyl ester was potent. Five milligrams of 10-carbomethoxymethyl-1,2-benzanthracene (m. p. 128°–130° C.), another of the compounds of Von Braun, was injected (August 20, 1937) into 10 strain A male mice; the same dose was repeated after 5 and after 8 months.

In the sixth month ulceration at the injection site was noted in 2 mice. The first 2 tumors developed at the end of 8 months. Tumors appeared in 5 of the 10 mice in 13 months. The experiment lasted for 16 months (table 5); in both of the animals dying at this time, 8 months after the last injection, the crystalline compound was seen at the injection site. In several of the mice the injected compound was seen at the margin of the tumor or in its interior.

One of the tumors obtained in 8 months was transplanted into six other strain A mice; the transplants grew progressively in all.

10-Hydroxy-1,2-benzanthracene (*fig. 2, formula XXI*).—Five milligrams of 10-hydroxy-1,2-benzanthracene was injected (February 12, 1937) into 20 mice of the C57 Brown strain (12 males and 8 females). The dose was repeated 4 times during the next 10 months. Slight ulceration occurred in 2 mice at the site of injection after 6 months. No tumors at the injection site were obtained in this experiment which was terminated in the fourteenth month (table 5).

10-Methoxy-1,2-benzanthracene (*fig. 2, for-*

mula XXII).—The 10-methoxyl derivative was injected (April 17, 1937) into 10 strain A and 10 C_3H male mice. The injection of 5 mg. was repeated after 3 and after 10 months.

Although the first tumor appeared within 4 months, tumor production was slow as can be seen from table 5. At the end of a year tumors had appeared in only 5 mice, at which time the other 15 mice were alive and negative. Tumors were obtained in a total of 10 mice by the time the experiment ended in the seventeenth month.

S-, Cl-, AND N-CONTAINING DERIVATIVES

10-Sulfhydrylmethyl-1,2-benzanthracene (*fig. 2, formula XXIII*).—This sulfur-containing analogue (m. p. 172°–173° C.) of 10-hydroxymethyl-1,2-benzanthracene, one of the compounds obtained from Von Braun, was injected (August 19, 1937) in a dose of 5 mg. into 10 strain A male mice. The injection was repeated after 5 and after 8 months. No tumors were obtained in this experiment, which lasted for 19 months, although 9 of the 10 mice were alive at 12 months, and 5 were still alive at 15 months (table 6).

10-Chloromethyl-1,2-benzanthracene (*fig. 2, formula XXIV*).—10-Chloromethyl-1,2-benzanthracene (m. p. 190°–191° C.), another compound obtained from Von Braun, was injected (August 19, 1937) into 10 strain A male mice in a dose of 5 mg. which was repeated after 5 and after 8 months.

Within a month after the injection, ulceration occurred at the site of injection in most of the mice. The ulcers healed, occurred again within a month after the second injection, and then healed again. The same effect was obtained after the third injection.

At the end of a year small papillomas appeared at the injection site in two mice. These, however, did not grow progressively. Histologically, they had not in-

TABLE 6.—*Sulfur-, chlorine- and nitrogen-containing derivatives related to 10-methyl-1,2-benzanthracene*

(Production of subcutaneous tumors in mice)

Substituent	Formula No.	Dose (crystals)	Mice			Total number of mice with tumors (+), and negative surviving mice (−) after various periods (in months)			
			Number	Sex	Strain	6 months	9 months	12 months	15 months
10-CH_2SH	XXIII	*Milligrams* 15	10	M	A	0+ 10−	0+ 10−	0+ 9−	0+ 5−
10-CH_2Cl	XXIV	15	10	M	A	0+ 10−	0+ 8−	0+ 4−	0+ 2−
10-CH_2CN	XXV	15	10	M	A	1+ 7−	2+ 5−	2+ 5−	1+ 1−
10-$CH_2N(CH_3)_2$	XXVI	15	10	M	A	0+ 9−	0+ 9−	0+ 9−	0+ 8−
10-$CH_2N(C_2H_5)_2$	XXVII	15	10	M	A	0+ 10−	0+ 10−	0+ 10−	0+ 3−
10-CN	XXVIII	7	20	M+F	A	0+ 20−	0+ 17−	0+ 12−	0+ 6−
10-NO_2	XXIX	22	20	M+F	A	0+ 20−	0+ 17−	0+ 5−	0+ 5−
10-NH_2	XXX	15	20	M+F	A	0+ 9−	1+ 4−	1+ 1−	
		6	40	M+F	A+M[1]	0+ 36−	0+ 35−	1+ 15−	1+ 5−

[1] The M (leaden) strain is also designated as L.

vaded the underlying tissues by the time the experiment ended in the sixteenth month (table 6).

10-Cyanomethyl-1,2-benzanthracene (fig. 2, formula XXV).—10- Cyanomethyl-1,2-benzanthracene (m. p. 172°–174° C.), also obtained from Von Braun, was injected (August 20, 1937) into 10 strain A male mice. The dose of 5 mg. was repeated after 5 and after 8 months. Ulceration developed at the injection site in 6 of the mice in the sixth month. The first tumor appeared at the base of an ulcer at the end of the sixth month.

A tumor obtained during the eighth month was found to contain the yellow compound in and around the tumor. Portions of this tumor, selected from areas distant from the compound, were implanted into six other strain A mice. Within 3 weeks all of the implants developed into large tumors.

Tumors were obtained in 4 of the 10 mice in this experiment which lasted for 19 months (table 6).

10-Dimethylaminomethyl-1,2-benzanthracene (fig. 2, formula XXVI).—10-Dimethylaminomethyl-1,2,benzanthracene (m. p. 110°–112° C.), obtained from Von Braun, was injected (August 20, 1937) into 10 strain A male mice. The 5-mg. dose was repeated after 5 and after 8 months.

No tumors were obtained at the injection site in this experiment, which was terminated in the seventeenth month, although 8 of the 10 mice were still alive after 15 months (table 6).

10-Diethylaminomethyl-1,2-benzanthracene (fig. 2, formula XXVII).—10-Diethylaminomethyl-1,2-benzanthracene (m. p. 81°–83° C.), the last of the 10 compounds obtained from Von Braun, was injected (August 20, 1937) into 10 strain A male mice in a dose of 5 mg., which was repeated after 5 and after 8 months.

All 10 of the mice were alive at the end of a year, but no tumors at the injection site were obtained in any of the animals by the time the experiment ended in the seventeenth month (table 6).

10-Cyano-1,2-benzanthracene (fig. 2, formula XXVIII).—10-Cyano-1,2-benzanthracene, synthesized by Dr. Hartwell in Professor Fieser's laboratory, was injected (October 17, 1938) into 10 male and 10 female mice of the A strain. The first injection of 2 mg., was followed by another of 5 mg., 5 months later.

No induced tumors were obtained at the injection site although 12 of the mice survived for a year. A glandular subcutaneous carcinoma which appeared in a female after 14 months was not classified as induced although the crystalline compound was seen adjacent to the tumor at autopsy.

The crystalline compound was seen at the injection site in all of the six survivors which were killed in the sixteenth month, 11 months after the last injection.

10-Nitro-1,2-benzanthracene (fig. 2, formula XXIX).—The 10-nitro derivative was injected (October 29, 1937) into 10 male and 10 female mice of the A strain. A total of 5 injections, usually of 5 mg., was given during the first 15 months.

During the fourth month ulceration occurred at the injection site in six of the females. No tumors were obtained at the site of injection in any of the mice by the time the last two survivors were killed in the twentieth month (table 6). The compound was seen in both of these mice at autopsy 5 months after the last injection.

10-Amino-1,2-benzanthracene (fig. 2, formula XXX).—Five milligrams of the 10-amino derivative was injected (October 29, 1937) into 10 male and 10 female mice of the A strain. The injection was repeated after 2 and after 6 months. Ulceration, which subsequently healed, occurred in some of the mice after each injection. A sarcoma developed in a male mouse at the injec-

tion site in the eighth month (table 6).

Since survival was poor in this experiment, it was repeated with 40 mice. One milligram was injected (January 7, 1939) into 10 male and 10 female mice of the A strain and into similar mice of the M (leaden) strain. Five injections in all were given.

Survival was better in this experiment which lasted for 18 months. The smaller dosage, however, did not yield an increased number of tumors in spite of the reduction in the ulceration. Only one induced tumor was obtained; this was a sarcoma which developed in a strain M (leaden) female mouse in the twelfth month.

This compound appears to be a very weak carcinogen since it produced sarcomas in but 2 of 60 mice.

COMPOUNDS RELATED TO 1,2,5,6-DIBENZANTHRACENE AND 3,4-BENZPYRENE

Four meso-substituted derivatives of 1,2-benzanthracene, which have the pentacyclic systems of 1,2,5,6-dibenzanthracene or of 3,4-benzpyrene, were examined for potency.

9-Methyl-1,2,5,6-dibenzanthracene (fig. 3, formula XXXI).—Only 1 injection of 2 mg. of 9-methyl-1,2,5,6-dibenzanthracene was given (March 16, 1939) to 10 male and 10 female mice of the A strain, for tumors developed so rapidly that all of the mice were dead in less than 4 months.

Ulceration at the site of injection was noted after 3 weeks; by the end of the second month ulceration was present in all of the mice. The first two tumors appeared early in the third month; by the end of 3 months tumors were present in 12 mice. Three of the mice died at this time, extensively ulcerated at the injection site; another mouse with an ulcer died during the fourth month. The remaining 4 mice developed tumors at the site of injection early in the fourth month.

This compound is a very potent carcinogen: it produced tumors in 16 of 20 mice in 3½ months. It would appear from this experiment that this compound ranks with methylcholanthracene in its ability to produce sarcomas rapidly (table 7).

5-Methyl-3,4-benzpyrene (fig. 3, formula XXXII).—5-Methyl-3, 4-benzpyrene was also found to be a most potent carcinogen, for after the injection (March 17, 1939) of 1 dose of 2 mg., tumors were obtained in 19 of 20 mice in less than 3½ months.

In this experiment 10 male and 10 female mice of the A strain were employed. Ulceration at the site of injection appeared in most of the mice before the end of the

TABLE 7.—*10-Substituted derivatives of 1,2-benzanthracene related to 1,2,5,6-dibenzanthracene and 3,4-benzpyrene*

[Production of subcutaneous tumors in mice]

Compound	Formula No.	Dose (crystals)	Mice			Total number of mice with tumors (+) and negative surviving mice (−), after various periods (in months)			
			Number	Sex	Strain	3 months	4 months	6 months	12 months
		Milligrams							
9-Methyl-1,2,5,6-dibenzanthracene	XXXI	2	20	M+F	A	12+ 5−	16+ 0−	.....	.....
5-Methyl-3,4-benzpyrene	XXXII	2	20	M+F	A	15+ 4−	19+ 0−	.....	.....
3,4-Benzpyrene-5-aldehyde.....	XXXIII	2	10	M	A	1+ 9−	4+ 6−	10+ 0−	.....
5-Hydroxy-3,4-benzpyrene.....	XXXIV	7	20	M+F	A	0+ 20−	0+ 20−	0+ 20−	0+ 13−

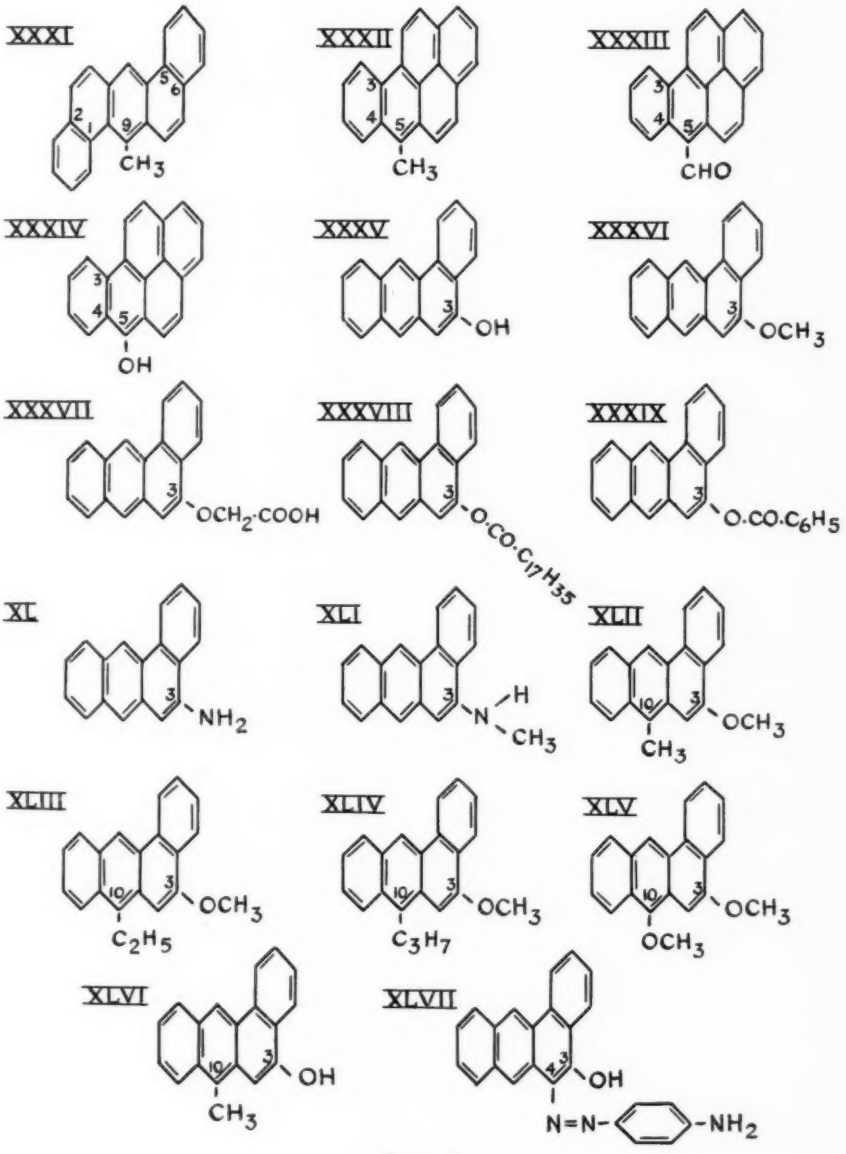
XXXI
XXXII
XXXIII
XXXIV
XXXV
XXXVI
XXXVII
XXXVIII
XXXIX
XL
XLI
XLII
XLIII
XLIV
XLV
XLVI
XLVII

FIGURE 3.

first month. Early in the third month tumors were present in 9 mice; tumors appeared in 6 more mice before the end of 3 months. The 4 survivors developed tumors early in the fourth month (table 7).

This hydrocarbon produced tumors even more rapidly than did 9-methyl-1,2,5,6-dibenzanthracene.

3,4-Benzpyrene-5-aldehyde (*fig. 3, formula XXXIII*).—Another 5-substituted benzpyrene, with an aldehyde instead of a methyl group, was injected (March 17, 1939) into 10 strain A male mice in a dose of 2 mg. Not enough of the compound was available for a larger number of mice.

This compound also proved to be carcinogenic. The first tumor appeared at the end of 3 months, and three more tumors developed during the fourth month. Ulceration occurred at the injection site in only two of the mice.

Tumors appeared in four mice during the fifth month. The two survivors developed tumors early in the sixth month. This aldehyde derivative thus produced tumors in all of the mice in less than 6 months (table 7).

5-Hydroxy-3,4-benzpyrene (*fig. 3, formula XXXIV*).—The 5-hydroxyl derivative of 3,4-benzpyrene was found to be inactive. Two milligrams were injected (October 17, 1938) into 10 male and 10 female mice of the A strain, and an injection of 5 mg. was given 6 months later.

Of the 20 mice, 19 were alive after 9 months and 13 lived for a year. The 6 survivors were killed and the experiment was terminated in the sixteenth month. The injected compound was seen in the subcutaneous tissues in all of these mice 9 months after the last injection; the original greenish-yellow color of the compound, however, was seen to have turned to black in the mice after a year.

Ulceration occurred in three of the mice during the course of the experiment. No tumors were produced at the injection site.

3-SUBSTITUTED DERIVATIVES

Rather early in the course of these studies, we desired to ascertain the carcinogenic activity of derivatives with certain polar groups. Although the results obtained with aromatic hydrocarbons were interesting, and in some cases striking, it was considered worth while to examine related compounds having substituents such as were present in naturally occurring phenanthrene derivatives. Since it was not possible at that time to obtain the desired compounds, the 3-hydroxyl and 3-methoxyl derivatives of 1,2-benzanthracene were injected into mice, inasmuch as they happened to be available from a previous investigation (*14*) even though negative results as regards carcinogenic action were anticipated in view of the report (*10*) from London that 3-methyl-1,2-benzanthracene was inactive.

3-Hydroxy-1,2-benzanthracene (*fig. 3, formula XXXV*).—About 10 mg. of the 3-hydroxyl derivative was injected (April 30, 1935) into 11 female strain A mice. Ulceration occurred at the injection site in 4 of the mice after about 6 months. No tumors were obtained in this experiment in which 8 of the 11 mice survived for a year, and 5 survived for 18 months. The experiment was terminated after 21 months at which time crystals of the injected compound were seen to be still present in the subcutaneous tissues.

The experiment was repeated (April 14, 1936), because a tumor had meanwhile been induced with the 3-methoxyl derivative. Ten strain dba male mice each received four injections, of 10, 10, 5, and 6 mg., respectively, over a period of 8 months. During the ninth month tumors developed in two of the mice at the injection site. The injected compound was present in the interior of both tumors.

TABLE 8.—*3-Substituted derivatives of 1,2-benzanthracene*

[Production of subcutaneous tumors in mice]

Substituent	Formula No.	Dose (crystals)	Mice			Total number of mice with tumors (+) and negative surviving mice (−), after various periods (in months)			
			Number	Sex	Strain	6 months	9 months	12 months	15 months
		Milligrams							
3-hydroxy	XXXV	10	11	F	A	0+ 10−	0+ 9−	0+ 8−	0+ 6−
		31	10	M	dba	0+ 10−	2+ 4−	2+ 1−	2+ 1−
3-methoxy	XXXVI	10	12	M+F	A+dba	0+ 9−	0+ 5−	1+ 0−	.....
		25	10	F	dba	1+ 9−	3+ 5−	4+ 2−	4+ 1−
3-carboxymethoxy	XXXVII	[1] 2	10	M	[2] M	0+ 10−	0+ 10−	0+ 6−	0+ 4−
3-hydroxy, stearate	XXXVIII	13	16	M	A	0+ 16−	0+ 16−	0+ 11−	0+ 9−
3-hydroxy, benzoate	XXXIX	20	20	M+F	dba	0+ 16−	0+ 13−	0+ 9−	.....
3-amino	XL	20	20	M+F	[2] M	0+ 17−	0+ 9−	0+ 3−	.....
3-methylamino	XLI	10	20	M+F	[2] M	0+ 16−	0+ 7−	0+ 3−	.....

[1] In solution in filtered lard.
[2] The M (leaden) strain is also designated as L.

Both sarcomas were transplanted for two transplant generations.

No tumors were induced at the injection site in any of the other mice by the time the experiment was terminated after 15 months (table 8).

3-Methoxy-1,2-benzanthracene (*fig. 3, formula XXXVI*).—Ten milligrams of the 3-methoxyl derivative was injected (April 30, 1935) into 12 mice; 4 were strain A females, and 8 were strain dba males. Ulceration occurred in 4 mice (in 3 of the A strain and in 1 of the dba strain) after several months and gradually increased in severity.

During the twelfth month a tumor developed at the injection site in a strain dba male. The compound was thereupon injected (April 14, 1936) into 10 strain dba female mice; 3 injections, of 10, 10, and 5 mg. were given over a period of 8 months. Ulceration occurred in several of the mice and, after 6 months, a sarcoma appeared at the injection site in 1 animal. This was transplanted for 5 transplant generations and then discontinued. During the ninth month, tumors appeared at the injection site in 2 mice; one of these developed at the margin of an ulcer. One of these tumors was also successfully transplanted.

Another sarcoma developed at the injection site in the eleventh month. In two of the five mice that survived for more than 9 months, mammary-gland tumors developed in regions distant from the injection site. These were not classified as induced tumors.

The single mouse that survived for 15 months bore two noninvasive papillomas on the skin over the injection site. Malignant tumors were obtained at the injection site in four of the mice in this experiment (table 8).

3-Carboxymethoxy-1,2-benzanthracene (fig. 3, formula XXXVII).—When it was unexpectedly found that the 3-hydroxyl and 3-methoxyl derivatvies were carcinogenic, a number of 3-substituted derivatives were examined for potency. Some of these had a substituent at the 10-position as well, but five of them had substituents only at the 3-position.

The small amount of the 3-carboxymethoxyl derivative that was available was dissolved in filtered lard in a concentration of 5 mg. per cubic centimeter. Ten strain M (leaden) male mice were given (February 12, 1937) 0.2 cc. of this solution; 3 months later the injection was repeated.

An ulcer with a thickened base developed at the injection site in one mouse after 9 months, but no tumors were obtained in any of the mice at the site of injection. The four survivors were killed after 16 months (table 8).

3-Hydroxy-1,2-benzanthracene stearate (fig. 3, formula XXXVIII).—The stearate of 3-hydroxy-1,2-benzanthracene was injected (June 19, 1936) into 16 strain A male mice. The initial injection of 8 mg. was followed 2 months later by a second injection of 5 mg. There was not enough of this compound available for a larger number of mice.

No tumors were obtained at the injection site in this experiment which was terminated after 15 months by killing the nine survivors. The compound was seen in the subcutaneous tissues in seven of the mice 13 months after the last injection (table 8).

3-Hydroxy-1,2-benzanthracene benzoate (fig. 3, formula XXXIX).—Ten milligrams of the benzoate of 3-hydroxy-1,2-benzanthracene was injected (April 14, 1936) into 10 male and 10 female mice of the dba strain. The injection was repeated after 3 months.

No tumors appeared at the site of injection in any of the mice in this experiment which lasted for more than 14 months (table 8). The compound was seen at the injection site in the animals which survived for 11 months after the last injection.

In several of the mice that died in the course of the experiment a fibrinous exudate was noted in the subcutaneous tissues at the injection site, or in the peritoneum, or in both areas.

3-Amino-1,2-benzanthracene (fig. 3, formula XL).—Ten milligrams of the 3-amino derivative was injected (February 12, 1937) into 10 male and 10 female mice of the M (leaden) strain; 5 mg. was injected after 3 months and again after 5 months.

Ulceration at the site of injection occurred in several mice, but no tumors developed. Survival was not very good; in this experiment, which lasted for 14 months, nine mice lived for 9 months, and only three lived for 12 months. This may possibly have been due to a toxic action of the compound, for one of the animals succumbed immediately after the second subcutaneous injection.

3-Methylamino-1,2-benzanthracene(fig. 3, formula XLI).—Five milligrams of the 3-methylamino derivative was injected (February 12, 1937) into 10 male and 10 female mice of the M (leaden) strain. The injection was repeated after 5 months.

Ulceration occurred in several of the mice after each injection; this healed slowly in most cases. No tumors were obtained at the injection site in any of the mice in this experiment which lasted for 14 months.

DISUBSTITUTED DERIVATIVES

3, 10-DISUBSTITUTED COMPOUNDS

When it was found that substitution at the 10-position conferred a high degree of potency, and that 3-substitution conferred a slight degree of potency, a series of 3,10-disubstituted derivatives were examined

TABLE 9.—*3,10-Disubstituted derivatives of 1,2-benzanthracene*
[Production of subcutaneous tumors in mice]

Substituents	Formula No.—	Dose (crystals)	Mice			Total number of mice with tumors (+), and surviving negative mice (−), after various periods (in months)			
			Number	Sex	Strain	9 months	12 months	15 months	18 months
		Milligrams							
3-Methoxy-10-methyl	XLII	[1] 3.5	20	F	A	1+	3+	3+	4+
		5				19−	15−	11−	3−
		10	6	M+F	A+C_3H	0+	1+	2+	.....
						6−	4−	0−	.....
3-Methoxy-10-ethyl	XLIII	[1] 3.5	20	F	A	1+	1+	1+	1+
		5				17−	17−	14−	9−
3-Methoxy-10-propyl	XLIV	[1] 3.5	20	M	A	0+	0+	0+	0+
		5				19−	18−	12−	2−
3,10-Dimethoxy	XLV	15	10	M	A	0+	0+	.....	.....
						9−	6−	.....	.....
3-Hydroxy-10-methyl	XLVI	5	20	M	A+C_3H	0+	0+	.....	.....
		3	10	M	C_3H	16−	8−	.....	.....
		[1] 1	27	M+F	C_3H+M[2]+A+dba				
3-Hydroxy-4-(p-aminophenylazo).	XLVII	28	20	M	A+dba	0+	0+	0+	.....
						16−	10−	8−	.....

[1] In solution in filtered lard.
[2] M (leaden) or L.

for carcinogenic activity to ascertain whether there would be an additive effect.

3-Methoxy-10-methyl-1,2-benzanthracene (*fig. 3, formula XLII*).—The 3-methoxy-10-methyl derivative was injected (December 17, 1936) into 20 strain A female mice. The original dose, of 0.2 cc. of a solution in filtered lard containing 5 mg. of the compound, was followed 3 months later by a second injection of 0.5 cc. of this solution; a third injection, 5 mg. of the crystalline compound, was given after 9 months.

Small areas of ulceration developed at the injection site in four mice. The first tumor developed in the ninth month; tumors appeared in two other mice at the end of a year. By the time the experiment was terminated after 18 months, tumors had been induced in four mice (table 9).

Six other animals, three C_3H and three A mice of both sexes, were each given one injection of 10 mg. of the crystalline compound. Tumors were induced at the injection site in the two C_3H males between the eleventh and fifteenth months.

This compound was thus found to be a weak carcinogen. The carcinogenic potency conferred on 1,2-benzanthracene by a methoxyl group at the 3-position or by a methyl group at the 10-position was not enhanced when both groups were introduced into the same molecule; on the contrary, the 3-methoxy-10-methyl compound was much less potent than 10-methyl-1,2-benzanthracene.

3-Methoxy-10-ethyl-1,2-benzanthracene (*fig. 3, formula XLIII*).—The 3-methoxy-10-ethyl derivative was administered (December 17, 1936) to 20 strain A female mice in the same way and in the same doses as the 3-methoxy-10-methyl analogue, i. e., a solution in lard was used for the first two injections, and the crystalline compound for the third (table 9).

A sarcoma at the injection site was pro-

duced in one mouse after 7 months. No other induced tumors were obtained although survival was excellent. The nine survivors were killed after 18 months.

While the introduction of a methoxyl group at the 3-position of 1,2-benzanthracene resulted in a compound possessing carcinogenic potency, a methoxyl group introduced at the 3-position of 10-ethyl-1,2-benzanthracene resulted in almost complete abolition of the high activity possessed by the latter compound.

3-Methoxy-10-propyl-1,2-benzanthracene (fig. 3, formula XLIV).—The 10-propyl analogue of the 2 preceding compounds was injected (December 22, 1936) into 20 strain A male mice in the same way (table 9).

Survival was good: 18 mice lived for more than a year, and 12 were alive after 15 months. No tumors were induced at the injection site. The addition of the 3-methoxyl group did not confer any potency on the inactive 10-propyl-1,2-benzanthracene; its introduction resulted in the abolition of the weak potency conferred by 3-methoxyl substitution in 1,2-benzanthracene.

3,10-Dimethoxy-1,2-benzanthracene (fig. 3, formula XLV).—Five milligrams of the 3,10-dimethoxyl derivative was injected (May 11, 1937) into 10 strain A male mice; the dose was repeated after 2 and after 9 months.

No tumors were obtained at the site of injection during the course of a year. The experiment was terminated in the thirteenth month by an epidemic infection.

A methoxyl group at the 10-position alone gave a compound of considerable potency, and a methoxyl group at the 3-position alone conferred a slight degree of potency; a combination of two methoxyl groups at the 3- and 10-positions in the same molecule resulted in abolition of the potency.

3-Hydroxy-10-methyl-1,2-benzanthracene (fig. 3, formula XLVI).—Five milligrams of the 3-hydroxy-10-methyl derivative was injected (April 17, 1937) into 10 strain A and 10 C_3H male mice. Within a few days 8 of the C_3H mice were dead and the other 2, which were sick, were killed. In all cases the organs, and the vessels in the subcutaneous tissues at the injection site, were congested: there was blood-tinged fluid in the thoracic cavity in many cases and fluid at the subcutaneous injection site in others. Three of the ten strain A mice also died in a few days, exhibiting similar changes.

A smaller dose of 3 mg. was next injected into 10 other C_3H males. Three days later 7 of these mice were dead, and 1 was dying; autopsy revealed the same changes as described above.

A solution of the compound was then made in filtered lard, in a concentration of 5 mg. per cubic centimeter. An injection of 0.2 cc. of this solution, containing 1 mg. of the compound, was given to 27 mice, of both sexes and of 4 strains (A, dba, M (leaden) and C_3H). Within 4 days 13 of the mice were dead; the dba strain mice were the only ones which did not suffer any casualties.

Only 23 of the 57 mice survived the first injection, and only 16 of these survived for 9 months. The experiment was terminated in the fourteenth month at which time the only mice alive were 4 of the strain A males which had received the largest dose (5 mg.). No tumors were obtained at the injection site in any of the mice (table 9).

Neither the 3-hydroxyl nor the 10-methyl derivatives was toxic. However, when these substituents were present in the same molecule, the resulting 3-hydroxy-10-methyl-1,2-benzanthracene was quite toxic

3-Hydroxy-4-(p-aminophenylazo)-1,2-benzanthracene (fig. 3, formula XLVII).—This di-

substituted derivative of 1,2-benzanthracene had a hydroxyl group at the 3-position and a p-aminophenylazo group at the 4-position. It was considered of interest, in view of the carcinogenic action on the liver of certain p-aminoazo compounds, to see what effect such a group would produce when introduced into the molecule of 1,2-benzanthracene.

This compound was injected (September 30, 1936) into 10 strain dba and 10 strain A male mice. Three injections of 1 mg. each, 5 injections of 3 mg. each, and 2 injections of 5 mg. each, were given over a period of 16 months. No tumors were obtained at the injection site although 8 of the mice survived for 15 months (table 9).

4- AND 5-SUBSTITUTED DERIVATIVES OF 10-METHYL-1,2-BENZANTHRACENE

In view of our previous finding that 10-methyl substitution in 1,2-benzanthracene conferred a high degree of potency for the production of sarcomas in mice, the effect of substitution in other positions of the molecule of 10-methyl-1,2-benzanthracene was explored in spite of the repressing effect of substitution in the 3-position described in the preceding section. As had been considered likely, it was found that some types of substitution enhanced, and others depressed, the activity of 10-methyl-1,2-benzanthracene.

4,10-Dimethyl-1,2-benzanthracene (fig. 4, formula XLVIII).—A solution was prepared containing 5 mg. of 4,10-dimethyl-1,2-benzanthracene per cubic centimeter of filtered lard; 0.2 cc. of this solution was injected (October 24, 1938) into each of 10 male and 10 female mice of the A strain.

The first tumor at the injection site appeared during the fifth month. By 6 months, tumors had been obtained in five mice, and by the end of the year tumors had been induced in a total of seven mice (table 10). The experiment was terminated in the fourteenth month.

The ulceration, which was such a prominent feature with 4,10-ace-1,2-benzanthracene (fig. 4, formula XLIX), both when a large dose of crystals and when 1 mg. in lard were given (*4*), was not obtained with the 4,10-dimethyl analogue. Moreover 1 mg. of the 4,10-dimethyl derivative produced tumors in a smaller proportion of the mice than did 0.1 mg. of the 4,10-ace derivative.

4,10-Dimethyl-1′, 2′, 3′, 4′-tetrahydro-1,2-benzanthracene (fig. 4, formula L).—Two milligrams of the 1′, 2′, 3′, 4′-tetrahydro derivative of 4,10-dimethyl-1,2-benzanthracene was injected (October 21, 1938) into each of 10 male and 10 female mice of the A strain; a second injection, of 3 mg., was given 6 months later.

No tumors at the injection site were obtained in any of the mice in this experiment which was terminated in the fourteenth month at which time three mice were still alive (table 10). The hydrocarbon was seen in the subcutaneous tissues in the mice that died 8 months after the last injection.

This tetrahydro 4,10-dimethyl derivative was less active than its tetrahydro-4,10-ace analogue (fig. 4, formula LI) which produced tumors (*4*) in 4 of 10 mice in 9 months. Thus, both the aromatic and hydrogenated 4,10-dimethyl derivatives were less active than their 4,10-ace analogues; apparently the five-membered ring confers higher potency than two methyl groups at these positions.

5-Cyano-10-methyl-1,2-benzanthracene (fig. 4, formula LIII).—Introduction of a methyl group at the 5-position markedly enhanced the potency of 10-methyl-1,2-benzanthracene, for the 5,10-dimethyl derivative (fig. 4, formula LII) was more rapidly acting (*4*) than the 10-methyl compound. Three

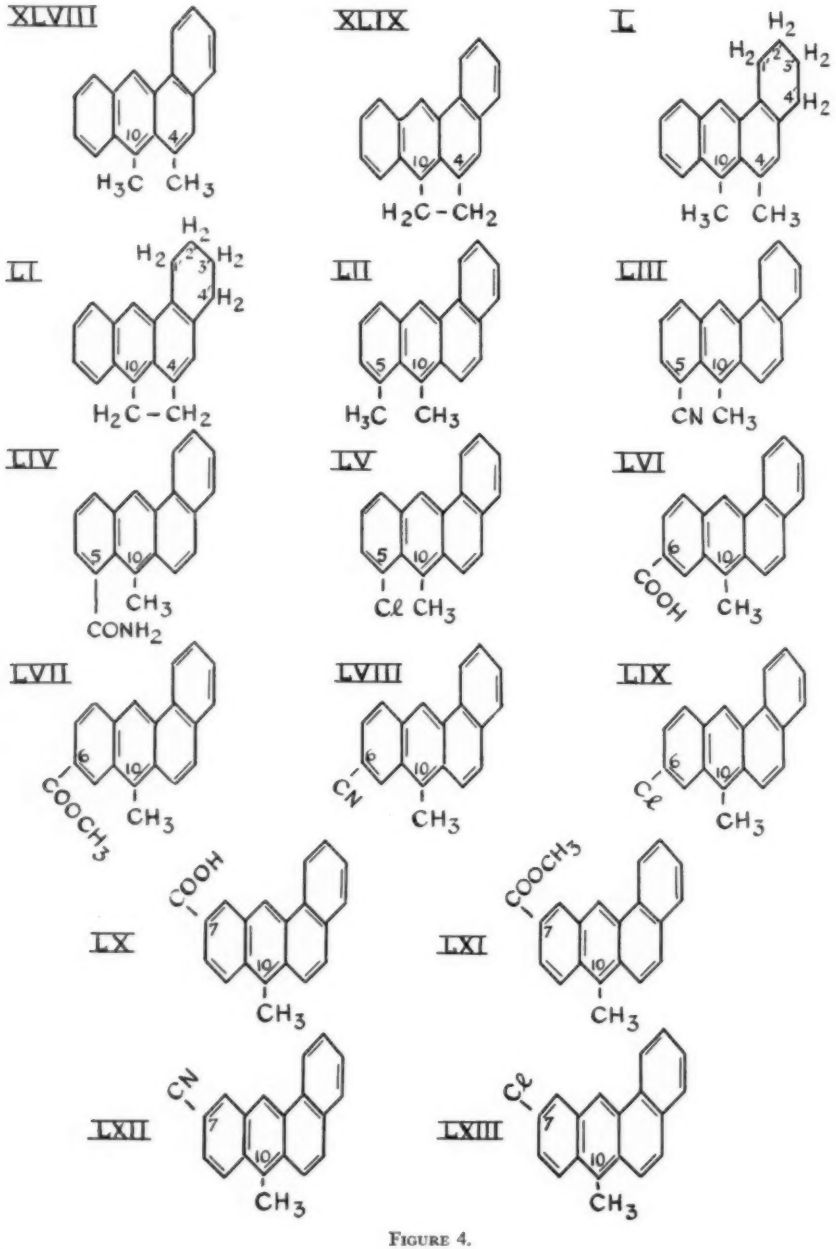
XLVIII
XLIX
L
LI
LII
LIII
LIV
LV
LVI
LVII
LVIII
LIX
LX
LXI
LXII
LXIII

FIGURE 4.

TABLE 10.—*4- and 5-Substituted derivatives of 10-methyl-1,2-benzanthracene*

[Production of subcutaneous tumors in mice]

Substituents	Formula No.	Dose (crystals)	Mice			Total number of mice with tumors (+), and surviving negative mice (−), after various periods (in months)			
			Number	Sex	Strain	4 months	6 months	9 months	12 months
		Milligrams							
4,10-Dimethyl	XLVIII	[1] 1	20	M+F	A	0+ 19−	5+ 10−	7+ 5−	7+ 3−
4,10-Dimethyl-1',2',3',4'-tetrahydro.	L	5	20	M+F	A	0+ 19−	0+ 18−	0+ 13−	0+ 6−
5-Cyano-10-methyl	LIII	5	10	M	C_3H	9+ 1−	10+ 0−		
5-$CONH_2$-10-methyl	LIV	2	20	M+F	A	5+ 14−	9+ 10−	14+ 4−	
5-Chloro-10-methyl	LV	[1] 2	20	M+F	A	1+ 19−	7+ 11−	16+ 1−	

[1] In solution in filtered lard.

compounds which had other substituents at the 5-position were consequently synthesized by Newman (*15*) at the Ohio State University.

Five milligrams of 5-cyano-10-methyl-1,2-benzanthracene was injected (July 29, 1937) into 10 C_3 H male mice. As was mentioned in previous communications (*1, 2*), this compound produced sarcomas very rapidly. By the end of the third month tumors were visible in 3 mice, and the small nodules which were palpable in 2 others rapidly grew larger soon after. During the fourth month tumors appeared in all but 1 of the remaining mice. The sole negative animal developed a tumor during the fifth month.

This compound apparently ranks with the cholanthrenes and 5,10-dimethyl-1,2-benzanthracene in potency.

Amide of 5-carboxy-10-methyl-1,2-benzanthracene (fig. 4, formula LIV).—Upon hydrolysis of the 5-cyano-10-methyl derivative, Newman (*15*), obtained the 5-$CONH_2$-10-methyl derivative. Two milligrams of this compound was injected (October 17, 1938) into each of 10 male and 10 female mice of the A strain.

As mentioned previously (*2*), this compound was quite potent. Ulceration at the site of injection was noted in 5 of the mice during the third and fourth months. A small tumor was palpated in 1 mouse during the third month and in another mouse early in the fourth month; at the end of 4 months tumors were present in 5 mice. Tumors were produced in a total of 9 and 14 mice after 6 and 9 months, respectively (table 10). When the negative survivors were killed in the eleventh month the compound was seen in the subcutaneous tissues at the injection site.

Sall and Shear (*7*) subsequently ascertained that subcutaneous tumors could be obtained in mice with as little as 0.1 mg. of this compound. This potent carcinogen is of considerable interest because it is not a hydrocarbon inasmuch as it contains the reactive $CONH_2$ group which is a component of many biologically occurring compounds.

5-Chloro-10-methyl-1,2-benzanthracene (fig. 4, formula LV).—A solution of the 5-chloro-10-methyl derivative, synthesized by Newman (*15*), was prepared containing 5 mg. of the compound per cubic centimeter of filtered lard; 0.2 cc. was injected (December 10, 1937) into 10 male and 10 female

mice of the A strain. The injection was repeated after 4 months.

As mentioned previously (*4, 2*), this was found to be a potent compound. The first tumor, which appeared at the end of the fourth month, was transplanted and grew in all of the new hosts. At the end of 6 months induced tumors had been obtained in 7 mice; one of these tumors was transplanted and grew in all of the recipients. When the experiment was terminated after 9 months, tumors had been induced in a total of 16 mice.

Thus 5-chloro substitution in the molecule of 10-methyl-1,2-benzanthracene gave a potent carcinogen with a moderately rapid rate of tumor production.

6- AND 7-SUBSTITUTED DERIVATIVES OF 10-METHYL-1,2-BENZANTHRACENE

Because of the interesting results obtained with 5-substituted derivatives of 10-methyl-1,2-benzanthracene, four 6-substituted and four 7-substituted analogues were synthesized by Newman and Orchin (*16, 17*) at the Ohio State University. The substituents introduced were the carboxyl, carbomethoxyl, cyano, and chloro groups.

6-Carboxy-10-methyl-1,2-benzanthracene (fig 4, formula LVI).—Two milligrams of the 6-carboxy-10-methyl compound was injected (October 22, 1938) into 10 male and 10 female mice of the A strain. Eleven mice survived for 6 months, when an intercurrent infection began to kill off many of the animals. The 4 sick survivors were killed in the tenth month. No tumors developed at the site of injection in this period (table 11).

6-Carbomethoxy-10-methyl-1,2-benzanthracene (fig. 4, formula LVII).—Two milligrams of the 6-carbomethoxy-10-methyl analogue was injected (October 22, 1938) into 20 strain A mice of both sexes. Survival was poor because of the same epidemic infection that affected the mice in the preceding experiment. No tumors developed at the injection site by the time the 3 sick sur-

TABLE 11.—*6- and 7-Substituted derivatives of 10-methyl-1,2-benzanthracene*

[Production of subcutaneous tumors in mice]

Substituents	Formula No.	Dose (crystals)	Mice			Total number of mice with tumors (+), and negative surviving mice (−), after various periods (in months)			
			Number	Sex	Strain	6 months	9 months	12 months	15 months
		Milligrams							
6-Carboxy-10-methyl	LVI	2	20	M+F	A	0+ 11−	0+ 5−		
6-Carbomethoxy-10-methyl	LVII	2	20	M+F	A	0+ 6−	0+ 3−		
6-Cyano-10-methyl	LVIII	2	20	M+F	A	0+ 8−			
6-Chloro-10-methyl	LIX	2	20	M+F	A	0+ 15−	0+ 8−		
7-Carboxy-10-methyl	LX	5	10	M	C_3H	0+ 9−	0+ 2−		
7-Carbomethoxy-10-methyl	LXI	5	10	M	A	0+ 6−	0+ 4−	0+ 1−	
7-Cyano-10-methyl	LXII	10	10	M	C_3H	2+ 8−	6+ 4−	7+ 2−	
7-Chloro-10-methyl	LXIII	10	10	M	A	0+ 9−	2+ 8−		
		2	20	M+F	A	2+ 18−	2+ 18−	4+ 11−	4+ 9−

vivors were killed in the tenth month (table 11).

6-Cyano-10-methyl-1,2-benzanthracene (fig. 4, formula LVIII).—Two milligrams of the 6-cyano-10-methyl analogue was injected (October 22, 1938) into 20 strain A mice of both sexes. The same epidemic infection, which affected chiefly the liver, brought this experiment also to an early close. The 2 sick survivors were killed at the end of 8 months by which time no tumors had developed at the injection site (table 11).

6-Chloro-10-methyl-1,2-benzanthracene (fig. 4, formula LIX).—Two milligrams of the 6-chloro-10-methyl analogue was injected (October 21, 1938) into 20 strain A mice of both sexes. Although the epidemic infection interfered with this experiment also, 8 of the mice surviced for 9 months. The experiment was terminated after 11 months by killing the 4 survivors, in all of which the crystalline compound was found at the site of injection. No tumors were obtained in this experiment (table 11).

7-Carboxy-10-methyl-1,2-benzanthracene (fig. 4, formula LX).—Five milligrams of the 7-carboxy-10-methyl analogue was injected (July 29, 1937) into the 10 male C_3H mice. Nine survived for 6 months, but only 2 were alive after 9 months because of an intercurrent infection. No tumors were obtained (table 11).

7-Carbomethoxy-10-methyl-1,2-benzanthracene (fig. 4, formula LXI).—Two milligrams of the 7-carbomethoxy-10-methyl analogue was injected (December 17, 1937) into 10 strain A male mice. Another injection of 3 mg. was given after 4 months. This experiment, also, was affected by an epidemic infection. Four mice survived for 9 months, and 1 mouse for a year. No tumors were obtained at the injection site (table 11).

7-Cyano-10-methyl-1,2-benzanthracene (fig. 4, formula LXII).—Five milligrams of the 7-cyano-10-methyl analogue was injected (July 29, 1937) into 10 C_3H male mice. The injection was repeated after 5 months. The first tumor was obtained at the end of 5 months; the second tumor appeared at the end of 6 months. Two tumors appeared during the eighth and two more during the ninth months. A tumor developed in another mouse in the tenth month. The two negative survivors died during the thirteenth month.

Tumors were thus obtained with this compound at the site of injection in 7 of the 10 mice in a period of 10 months (table 11).

7-Chloro-10-methyl-1,2-benzanthracene (fig. 4, formula LXIII).—Five milligrams of the 7-chloro-10-methy analogue was injected (June 14, 1937) into 10 strain A male mice. The injection was repeated after 5 months.

The first tumor appeared in the seventh month, and another developed during the eighth month. The other eight mice were still alive after 9 months, but the experiment was brought to an abrupt close in the tenth month by an epidemic infection.

Since tumors had been obtained at the injection site in 2 of the 10 mice, an additional supply of this compound was obtained, and was injected (October 22, 1938) into 20 strain A mice of both sexes, in a dose of 2 mg. The first 2 tumors at the injection site appeared during the sixth month. One tumor appeared during each of the following months: tenth, eleventh, and eighteenth. Thus, tumors developed at the site of injection in 5 of 20 mice in this experiment which lasted for 18 months (table 11).

9,10-DISUBSTITUTED DERIVATIVES OF 1,2-BENZANTHRACENE

In a previous communication (*4*) it was reported that substitution of a methyl group at 9-, the other meso position of 1,2-benzanthracene, gave a compound (fig. 5, formula LXIV) of high potency

TABLE 12.—*9,10-Disubstituted derivatives of 1,2-benzanthracene*

[Production of subcutaneous tumors in mice]

Substituents	Formula No.	Dose (crystals)	Mice			Total number of mice with tumors (+) and negative surviving mice (−), after various periods (in months)				
			Number	Sex	Strain	4 months	6 months	9 months	12 months	15 months
		Milligrams								
9,10-Dimethyl	XLVI	[1] 1	20	M+F	A	2+ 6−	3+ 4−	4+ 2−		
		[1] .35	20	M+F	A	6+ 13−	10+ 5−	15+ 0−		
9,10-Dimethoxy-9,10-dimethyl-9,10-dihydro	LXVII	15	20	M	C$_3$H	0+ 20−	0+ 19−	0+ 17−	0+ 16−	0+ 4−
9-Acetoxy-10-methyl	XLVIII	8.5	20	M+F	A	0+ 20−	0+ 18−	0+ 18−	1+ 8−	1+ 4−
9,10-Quinone	LXIX	15	10	M	C$_3$H	0+ 10−	0+ 10−	0+ 10−	0+ 7−	
9,10-Hydroquinone diacetate	LXX	15	10	M	C$_3$H	0+ 9−	0+ 6−	0+ 5−	0+ 3−	
1′,10-Dimethyl	LXXI	7	20	M+F	A	0+ 18−	0+ 17−	0+ 15−	0+ 14−	0+ 6−
		1.5 5	20	M+F	A	0+ 20−	0+ 20−	0+ 19−	0+ 14−	0+ 9−

[1] In solution in filtered lard.

which produced tumors in 17 of 20 mice in 9 months. When another methyl group was introduced at the 5-position, it greatly accelerated the action of 9-methyl substitution, for the 5,9-dimethyl derivative (fig. 5, formula LXV) produced tumors very rapidly, i. e., it produced tumors in 18 of 20 mice in 4 months.

It was therefore of interest to see what effect dimethyl substitution at both of the meso positions would have. Other compounds, also having substituents at both meso positions, were also examined for potency.

9,10-Dimethyl-1,2-benzanthracene (fig. 5, formula LXVI).—The earlier results obtained with the 9,10-dimethyl derivative, synthesized by Newman (*18*), were reported (*4*) in paper V of this series. Twenty strain A mice of both sexes received (December 10, 1937) 0.2 cc. of solution of filtered lard containing 5 mg. of the hydrocarbon per cubic centimeter. By the end of the first month severe ulceration had occurred in most of the mice at the injection site. The mice became thin and remained small. The compound, in this dose of 1 mg., was apparently toxic, for only eight mice survived for 4 months. Tumors appeared in two mice at the injection site by the end of 4 months, and in a total of five mice by the time the experiment terminated in the eleventh month (table 12).

The experiment was repeated, using a solution of one-tenth the concentration. Twenty strain A mice of both sexes received (January 2, 1938) 0.2 cc. of this more dilute solution, i. e., 0.1 mg. of the hydrocarbon; 3 months later an additional 0.25 mg. was given. The smaller dose produced much less local and general ill effects, for 18 of the 20 mice were alive and in good condition after 3 months, at which time ulceration was present only in 2. The first tumor appeared during the third month. By the end of 4 months tumors had been produced in 6 mice.

Four more tumors developed during each of the next 2 months. The last survivor developed a tumor during the eighth month, making a total of tumors in 15 of 20 mice in 8 months (table 12). Two of these tumors were successfully transplanted.

The 9,10-dimethyl derivative is, therefore, a potent carcinogen. This compound is another instance in which local and general damage were produced by the usual dose, with a concomitant poor yield of tumor production, and in which reduction of the dosage produced less damage and an increased tumor production.

Subsequently, Bachmann, Kennaway, and Kennaway (*19*) reported that this compound produced epitheliomas in mice, with the skin-painting technique, in a very short time. They, too, found that relatively large doses (1 to 10 mg.) produced severe ulceration and early death when injected subcutaneously in mice; no sarcomas were obtained in the animals which received doses of 2.5, 5, or 10 mg. of the hydrocarbon. Sall and Shear (*7*) have found that subcutaneous tumors may be obtained by a single injection of as little as 0.05 mg. of this hydrocarbon.

Law and Lewisohn (*20*) observed a generalized lymphomatosis in mice of the dba strain following skin painting with this compound.

9,10-Dimethoxy-9,10-dimethyl-9,10-dihydro-1,2-benzanthracene (*fig. 5, formula LXVII*).—Five milligrams of the 9,10-dimethoxy-9,10-dimethyl-9,10-dihydro derivative (*21*) prepared by Dr. M. S. Newman was injected (March 16, 1937) into 20 C_3H male mice. The injection was repeated after 4 and after 9 months.

Sixteen of the mice survived for a year, and 10 lived into the fifteenth month. No tumors developed at the injection site in any of the mice by the time the experiment was terminated after 18 months (table 12).

9-Acetoxy-10-methyl-1,2-benzanthracene (*fig. 5, formula LXVIII*).—The 9-acetoxy-10-methyl derivative was injected (October 29, 1937) into 20 strain A mice of both sexes in a dose of 5 mg.; another injection, of 3.5 mg., was given 5 months later. During the third month ulceration appeared at the site of injection in 2 mice, a thickened ulcer was present at the injection site in a mouse that died in the tenth month, and a sarcoma developed at the ulcer at the injection site in another mouse at the end of a year. No other tumors developed at the site of injection by the time the experiment was terminated after 17 months (table 12).

1,2-Benzanthraquinone (*fig. 5, formula LXIX*).—A specimen of 1,2-benzanthraquinone, which has an oxygen substituent at the 9- and 10- positions, was obtained from Dr. M. S. Newman. Five milligrams was injected (March 16, 1937) into 10 C_3H male mice, and the injection was repeated after 4 and after 9 months. All 10 of the mice were alive at 9 months, and 7 survived for a year. No tumors developed at the injection site in any of the animals by the time the last 3 survivors were killed after 14 months (table 12).

1,2-Benzanthrahydroquinone diacetate (*fig. 5, formula LXX*).—The 9,10-diacetoxy derivative, synthesized by Newman,[7] was injected (March 16, 1937) into 10 C_3H male mice. The dose of 5 mg. was repeated after 4 and after 9 months. Half of the mice lived for 9 months; the 3 survivors were killed in the thirteenth month. No tumors were obtained at the site of injection (table 12).

1′,10-Dimethyl-1,2-benzanthracene (*fig. 5, formula LXXI*).—The 1′,10-dimethyl derivative was injected (November 4, 1937) into 20 strain A mice of both sexes. The initial injection of 5 mg. was followed 5 months later by another of 2 mg. Although

[7] *Newman, M. S.: Unpublished data (1937).*

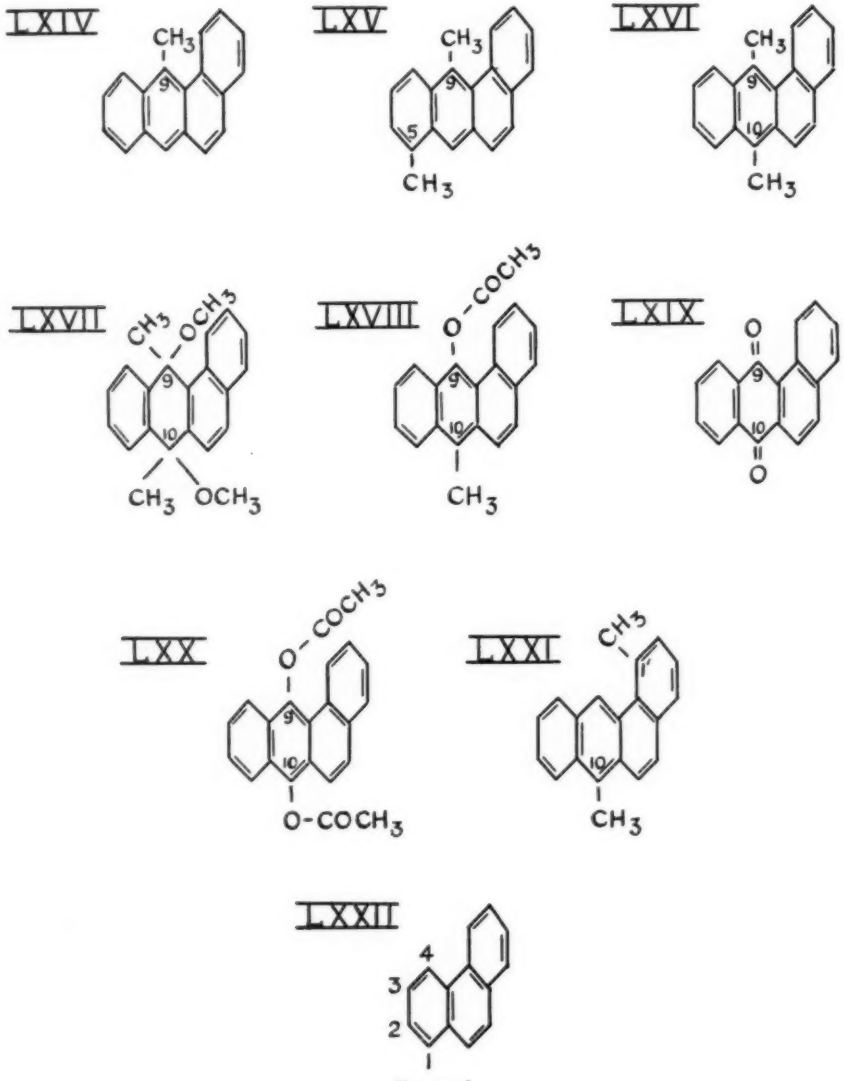
LXIV
CH3
9
LXV
CH3
9
5
CH3
LXVI
CH3
9
10
CH3
LXVII
CH3
OCH3
9
10
CH3
OCH3
LXVIII
O-COCH3
9
10
CH3
LXIX
O
9
10
O
LXX
O-COCH3
9
10
O-COCH3
LXXI
CH3
1'
10
CH3
LXXII
4
3
2
1

FIGURE 5.

ulceration occurred early in 5 of the mice at the injection site, no subcutaneous tumors developed; the ulcers healed after a few weeks. Fourteen of the mice survived for a year, and 6 lived for 15 months. The last 2 mice were killed in the twentieth month (table 12).

The experiment with this compound was repeated with a smaller dose. Twenty strain A mice of both sexes received (January 27, 1938) 0.25 cc. of a solution containing 2 mg. of the hydrocarbon per cubic centimeter of filtered lard; 5 months later the mice received 5 mg. of the crystalline compound. Fourteen mice survived for a year, and nine lived for 15 months. The last five survivors were killed in the seventeenth month (table 12). No tumors were induced in any of the mice at the injection site.

DISCUSSION

The ability of some polycyclic hydrocarbons to produce malignant tumors rapidly in a high proportion of the experimental animals, and in many tissues, aroused widespread interest. Indeed, their action was so striking that in some quarters there appeared to be a tendency to exaggerate the significance of the data obtained and, in view of the structure of these compounds, to imply that spontaneous tumors are caused by similar compounds. The following observations were therefore made:

> It may well be that substances analogous to the synthetic compounds found to be carcinogenic in London and in this laboratory are involved in human carcinogenesis, but this is at present only a conjecture (*4, p. 530*).
>
> Interesting as these synthetic compounds are, they are for the most part derivatives of benzanthracene, a structure not as yet demonstrated to be present in any naturally occurring material of biological origin (*2, p. 224*).

Nevertheless, these compounds have already proved to be useful tools in various types of studies in experimental cancer. Furthermore, the variations in potency produced by relatively minor alterations in structure may be found to have a significance transcending the artificial compounds themselves. If it should be found in the future that certain biologically occurring polycyclic compounds may be transformed, in the body, into others with carcinogenic properties, it would be of value to have available a systematic body of data on the effect of substituents, and of variations in the positions substituted, upon the carcinogenic potency of related molecules.

From the results given by the foregoing and other compounds, it would appear that, in general, subcutaneous injection has several advantages over the skin-painting technique if only one routine procedure for assaying the potency of large numbers of compounds is employed. In the analogous large-scale work in London, painting of the skin twice weekly was the routine; in this laboratory, subcutaneous injection was the usual procedure. We found that one injection sufficed in the case of the rapidly acting compounds. It was only with the negative or slowly acting compounds that the injection was repeated; even in these cases, however, it was repeated only a few times, at intervals of several months.

The subcutaneous-injection technique was employed here because the amount of time and labor necessary for painting the large numbers of mice involved in the assaying of several hundred compounds far exceeded the facilities of this laboratory. It was, therefore, gratifying to find, when the same compounds were assayed by both techniques, that both the cutaneous and subcutaneous tissues either gave similar results or that the latter were, with very few exceptions, more sensitive to the carcinogenic action of these compounds than was the skin. This was found to be

the case, for example, with the 10-methyl, 7-methyl, 3-methyl, and 4-methyl derivatives of 1,2-benzanthracene. The case of the 10-methyl compound has already been discussed (p. 306). With the 7-methyl derivative, the London group (*10*) obtained negative results in a skin-painting experiment, whereas we (*4*) obtained a low incidence of sarcoma production by subcutaneous injection. Subsequently Haddow (*22, 23*) stated that Kennaway also found this compound to be weakly carcinogenic when injected subcutaneously. (Cf. Cook and Kennaway (*3, p. 421*)). Although it had been found by the London workers (*10*) that the 3-methyl derivative gave negative results in a painting experiment, we obtained, following subcutaneous injection, positive results with the 3-hydroxyl and 3-methoxyl derivatives in spite of the fact that these groups have so far proven to be less potent than the methyl group. The London workers, according to Haddow (*23*) and Orr (*24*), later obtained sarcomas in mice with the 3-methyl compound following subcutaneous injection.[8] Similarly, painting of the skin with the 4-methyl compound gave no epitheliomas in any of 10 mice, according to Barry and others (*10*), whereas by the subcutaneous-injection technique, we obtained tumors in 15 of 20 mice in 10 months. The relatively rapid rate of sarcoma production by the 4-methyl derivative is indicated by the fact that the first 3 tumors appeared early in the fourth month (*4*).

The significance of these findings is appreciated when it is realized that both the 3-methyl and the 7-methyl derivatives had been employed in several laboratories abroad as noncarcinogenic control compounds in investigations in which the action of carcinogenic hydrocarbons was being studied. They emphasize anew the hazards involved in too-sweeping generalizations from limited data.

This is not to imply that the subcutaneous-injection technique is to be preferred to skin-painting on the ground that the former is always a more sensitive method. On the contrary, as has already been indicated (p. 327), the 9,10-dimethyl derivative is an instance of a compound which apparently is more potent for the skin than for the subcutaneous tissues of the mouse. These observations demonstrate the necessity, in interpreting the data of such experiments, of bearing in mind the uncertainties attendant upon extension of the conclusions to conditions other than those tested.[9]

The compounds which did not produce tumors at the site of injection are not necessarily to be regarded as noncarcinogenic. We found that they did not produce subcutaneous tumors under the conditions of these experiments. It is conceivable that lengthening the experimental period, increasing the number of animals used, or modifying the dose, the vehicle, the diet, the site of application, etc., might yield positive instead of negative results in some instances. For example, if the only data available for 20-methylcholanthrene were those from experiments in which this hydrocarbon had been implanted directly in the liver (mouse and rat) without resulting in demonstrable tumor production (*26*), the high carcinogenic potency of methylcholanthrene for other tissues of the mouse and the rat could hardly have been deduced.

The importance of using large numbers of animals in attempting to ascertain which compounds, under the conditions employed, are inactive and which are weakly carcinogenic also requires emphasis. For example, although the 3-methyl deriv-

[8] *See also sentence at bottom of next column, and top of p. 331.*

[9] *A more extended discussion of these considerations is given in a recent paper from this Institute by Shimkin (25).*

ative gave no tumors in a skin-painting experiment (*10*) in London in which 14 mice were employed, Cook and Kennaway have recently reported (*3*) obtaining 2 epitheliomas and 2 papillomas when 128 mice were painted with the same compound. It may also be pointed out, however, that such large numbers of mice are ordinarily required only when fine distinctions in potency are desired.

The negative results obtained with many compounds in these experiments do not appear to be ascribable to rapid absorption or removal of the injected material. Numerous instances have been noted in which the crystalline compounds were seen at autopsy still present at the site of injection 6 to 12 months or more after the administration of the last dose. Since, in most cases, the compounds were injected in the crystalline state their behavior was not complicated by the effects contributed by solvents used as vehicles.

It is also pointed out that the amount employed in the routine testing of new compounds is arbitrary. The London workers ordinarily employed a 0.3-percent solution in benzene in their skin-painting experiments. We originally employed 10 mg. of the crystalline compound for the first injection; this was reduced to 5 mg. in subsequent experiments. In recent years we have further reduced it to 2 mg. inasmuch as large doses were less effective than small doses in the case of some of the more potent compounds.[10] If, after about 3 months, no tumors had appeared, the injection was repeated, either in the same or in a larger dose.

The progress which had been made so rapidly in London in determining which structural features of benzanthracene derivatives are responsible for their potency has continued as new data have been accumulated.

In 1935 the London workers stated (*10*) that all simple derivatives of 1,2-benzanthracene which had no substituent in positions 5 or 6 had, up to that time, exhibited no capacity to produce malignant tumors. Their findings, not only with simple derivatives of 1,2-benzanthracene but also with 1,2,5,6-dibenzanthracene and 20-methylcholanthrene, led them to the generalization that substituents in the 5- and the 6-position of the benzanthracene molecule are necessary for a high order of carcinogenic activity; 3,4-benzpyrene was recognized (*10*, *27*) as not conforming to this generalization. The potency of 20-methylcholanthrene, in the light of the results obtained in London, appeared to them (*9*) to be determined mainly by the fact that it is a 5,6-disubstituted derivative of 1,2-benzanthracene.

The first evidence that substitution at the 6-position was not essential for high activity was obtained when we observed (*28*, *29*) that cholanthrene produced sarcomas in mice about as rapidly as methylcholanthrene. Similar results were subsequently obtained (*30*, *9*) in a skin-painting experiment by the London workers who agreed (*27*) that the methyl group at the 6-position is of no great significance in determining the potency of methylcholanthrene, but concluded that the essential structural feature is the pentacyclic system in the molecule.

However, when it was found (*4*) in this laboratory that 5,10-dimethyl-1,2-benzanthracene produced sarcomas about as rapidly as 20-methylcholanthrene, it became clear that the pentacyclic system is not essential for high potency. In fact, simple substitution of a single methyl group at the 10-position of 1,2-benzanthracene

[10] *Bachmann, Kennaway, and Kennaway (19) recently obtained analogous results on injecting the 9,10-dimethyl derivative subcutaneously. They also obtained, in skin-painting experiments with 5,9,10-trimethyl-1,2-benzanthracene, better tumor production with a 0.06-percent than with a 0.3-percent solution of this hydrocarbon.*

gives a compound which produced sarcomas almost as rapidly as did the cholanthrenes (*4*).

The London workers had previously examined two 10-substituted 1,2-benzanthracenes, but neither of them had exhibited any potency. This led them to the conclusion (*27*) that the introduction of substituents into positions, of the benzanthracene ring system, other than 5 and 6 is not only ineffective in stimulating the carcinogenic activity of the molecule, but may actually depress the activity of a carcinogenic derivative of benzanthracene. This generalization, based upon their extensive earlier data, was revised when it was found here (*4*) that meso substitution of a single methyl group was quite effective, for 9-methyl-1,2-benzanthracene is also a potent carcinogen, although more slowly acting than the 10-methyl derivative. Moreover, substitution of another methyl group at either meso position of 5-methyl-1,2-benzanthracene markedly enhances the potency; both the 5,9-dimethyl and 5,10-dimethyl derivatives were found in experiments in this laboratory (*4*) to be much more potent than the 5-methyl.

Although the 9,10-dimethyl compound did not prove to be very rapidly acting in our subcutaneous-injection experiments (*4*), it was quite potent. Bachmann, Kennaway, and Kennaway (*19*) more recently have found it to be the most rapidly acting carcinogen for the skin so far obtained. These results confirm the importance of meso substitution in the benzanthracene molecule. The potency of 3,4-benzpyrene was difficult to explain previously, since it was an exception to the generalization of the requirement of substitution at positions 5 or 6. In view of the significance of meso substitution, however, it may be that the high potency of benzpyrene is due to its being a derivative of 9-methyl-1,2-benzanthracene which is a potent carcinogen.

According to Cook and Kennaway (*3*), the high activity shown by the 9,10-dimethyl derivative, when applied to the skin of the mouse, supports the view that the effect of introducing substituents into the benzanthracene molecule is a summation effect in promoting carcinogenic activity, for both the 9-methyl and the 10-methyl compounds are compounds of fairly high potency. While such summation of potency apparently occurs in this and some other compounds such as the 5,9-dimethyl and 5,10-dimethyl derivatives, it does not hold for other compounds such as the 1′,10-dimethyl, 4,10-dimethyl, 3-methoxy-10-methyl, and a number of other derivatives of 10-methyl-1,2-benzanthracene (tables 9, 10, 11, and 12), and for other compounds examined in London. Whether summation or diminution of potency occurs upon combination, in the same molecule, of two active groups in the same positions which give potent compounds when introduced separately, has been unpredictable, and apparently depends on highly specific properties.

Further attesting to the importance of meso substitution in 1,2-benzanthracene are the results with 3 of the 4 compounds given in table 7. Introduction of a methyl group at the 9-position of 1,2,5,6-dibenzanthracene gives a compound [11] which was more potent than either 1,2,5,6-dibenzanthracene or 10-methyl-1,2-benzanthracene. This finding appears to require modification of another generalization. On the basis of the examination of some 20 simple derivatives of 1,2,5,6-dibenzanthracene the London group (*10*) stated—"the results fully justify the preliminary conclusion (Cook, 1932, *a*) that substitution, particularly in the *meso* positions, tends to

[11] *Positions 9 and 10 in the 1,2,5,6-dibenzanthracene molecule are interchangeable. For the relationship to 10-methyl-1,2-benzanthracene, compare formula I (fig. 1) with formula XXXI (fig. 3).*

diminish the carcinogenic potency of the 1 : 2 : 5 : 6 - dibenzanthracene molecule." Although no compounds had been examined in which a single alkyl group was attached to a meso position of 1,2,5,6-dibeznanthracene, their data suggested to them that "the property of carcinogenesis is facilitated (although not determined) by the presence of unsubstituted hydrogen atoms in the *meso* positions." However, the data given for compound XXXI in table 7 shows that meso substitution of a methyl group enhanced the potency of 1,2,5,6-dibenzanthracene.

The influence of meso substitution in enhancing carcinogenic potency is shown not only with 1,2-benzanthracene and with 1,2,5,6-dibenzanthracene, but with 3,4-benzpyrene as well. From the data in table 7, it is seen that 5-methyl-3,4-benzpyrene, which may be regarded as 1′,9-disubstituted derivative of 1,2-benzanthracene with a methyl group at position 10 (fig. 3, formula XXXII), is a highly potent, rapidly acting carcinogen. It produced tumors even more rapidly than did 3,4-benzpyrene itself. Meso substitution of appropriate groups is apparently so effective that introduction of an aldehyde group at the unsubstituted meso position of 3,4-benzpyrene gave a compound of high potency (table 7, formula XXXIII).

After finding that a single methyl group is more effective when introduced at position 10 of 1,2-benzanthracene than at any other position, several series of closely related compounds were examined. One of these, summarized in table 3, consists of simple hydrocarbon substituents ranging from a single carbon (methyl group) to a 5-carbon chain (amyl group). The formulas of these derivatives are shown in figure 1. The 10-methyl derivative was potent and rapidly acting, the 10-ethyl was potent and slowly acting, while the 10-propyl, 10-butyl, and 10-amyl derivatives were inactive. Such series of closely related compounds, with wide variations in potency, may be of value in studies of mechanisms, and of correlations between the properties of compounds and their carcinogenic potency.

Of considerable interest are the derivatives of 10-methyl-1,2-benzanthracene with polar substituents at the methyl carbon. Among the compounds shown in table 5 and figure 2, the 10-CH_2OH, the 10-$CH_2 \cdot COOCH_3$, the 10-$CH_2O \cdot COCH_3$, and the 10-CHO derivatives produced sarcomas. In these experiments the latter two compounds displayed an unexpectedly high potency. From the point of view of the possible existence of biochemical compounds with carcinogenic properties, such benzanthracene derivatives with polar substituents of types that are present in biologically occurring compounds may be of greater significance than hydrocarbons.

The methoxyl group also occurs in biological compounds of importance, and it is of interest that the 10-OCH_3 derivative is also potent (table 5).

The results obtained with previously examined compounds led the London workers (*9*) to point out the effect of the hydroxyl group in suppressing carcinogenic activity. While this is true in some instances, several hydroxylated compounds have been found in this laboratory to be carcinogenic; viz, the 3-OH and the 10-CH_2OH derivatives of 1,2-benzanthracene, and 15-hydroxy-20-methylcholanthrene. The question arises as to whether the alcoholic hydroxyl group, in appropriate positions, is more favorable for conferring carcinogenic potency than the phenolic hydroxyl group. In this connection it may be pointed out that none of the compounds with carboxyl groups have produced tumors in this laboratory, whereas the methyl ester was potent in one case (table 5, formula XX). In two

instances in which the hydroxyl group did not confer potency (10-OH and 3-OH-10-CH_3 derivatives of 1,2-benzanthracene) the corresponding methoxy derivatives were carcinogenic.

Among the unexpected findings was the display of potency by several chloro and cyano derivatives. The 5-cyano-10-methyl derivative acted about as rapidly as the cholanthrenes. The rather high potency of the 5-$CONH_2$-10-methyl derivative is of particular interest because of the presence of the $CONH_2$ group. Another unexpected result was the high toxicity of 3-OH-10-CH_3-1,2-benzanthracene, inasmuch as neither the 3-OH nor the 10-CH_3 derivative had displayed such toxicity on subcutaneous injection. However, the latter compound was apparently toxic when applied to the skin. On the other hand, the 9,10-dimethyl derivative was apparently toxic upon subcutaneous injection but not in the skin-painting work reported from London. From the limited data available, it would appear that when toxic effects have been noted, the carcinogenic potency has been low or absent.

The newer results with simple derivatives of 1,2-benzanthracene, particularly those having substituents at the meso positions, taken in conjunction with the positive results obtained in London (*9*) with 3,4-benzphenanthrene and 2-methyl-3,4-benzphenanthrene, raised the question in our minds several years ago as to whether it is the anthracene or the phenanthrene ring system that is more responsible for the carcinogenic potency of 1,2-benzanthracene. Most of the compounds found to be carcinogenic up to now may be considered as di-, tri-, or tetrasubstituted phenanthrene derivatives with substituents at the numbered positions shown in formula LXXII (fig. 5). Attempts were made to obtain a series of suitably substituted phenanthrene derivatives but the only one tested here so far, a trimethylphenanthrene with a melting point of 148.3°–149.3° C., proved to be negative (*2*). However, the synthesis of such derivatives was undertaken in this laboratory some time ago, and is now in progress.[12] If compounds of this type should be found to be carcinogenic, the subject would appear to have greater implications for biochemistry inasmuch as living organisms, in which benzanthracene compounds have not been demonstrated, contain many phenanthrene derivatives.

SUMMARY

Sixty-six 3-substituted and 10-substituted derivatives of 1,2-benzanthracene were examined for carcinogenic potency. The compounds were administered, in most instances by subcutaneous injection of the crystalline substances, to a total of about 1,300 mice of inbred strains (chiefly strain A).

Carcinogenic activity was exhibited by 24 of the 66 compounds.

The importance of meso substitution in contributing to the carcinogenic potency of derivatives of 1,2-benzanthracene, previously indicated by the activity of the 10-methyl, the 5,10-dimethyl, the 9-methyl, and the 5,9-dimethyl derivatives, was further supported by the activity shown by an additional series of potent 10-substituted derivatives of 1,2-benzanthracene. Furthermore, tumors were produced by 9-methyl-1,2,5,6-dibenzanthracene much more rapidly than by 1,2,5,6-dibenzanthracene; and 5-methyl-3,4-benzpyrene acted even more rapidly than did 3,4-benzpyrene.

High potency was exhibited by the 5-cyano-10-methyl derivative, and lesser potency by the 10-CH_2CN and the 7-

[12] *Hewett (31) has recently reported work along similar lines.*

cyano-10-methyl derivatives of 1,2-benz anthracene. The 5-chloro and the 7-chloro derivatives of 10-methyl-1,2-benzanthracene were also carcinogenic.

Of particular interest was the fact that a considerable number of oxygen-containing meso-substituted derivatives were potent-viz, the 10-CH_2OH, the 10-$CH_2O.COCH_3$, the 10-CHO, the 10-$CH_2.COOCH_3$, the 10-OCH_3, and the 5-$CONH_2$-10-methyl derivatives of 1,2-benzanthracene, and the 5-aldehyde of 3,4-benzpyrene.

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Retardation of Growth of the Rat Ingesting *p*-Dimethylaminoazobenzene (Butter Yellow)

I. The Effect of Various Dietary Supplements [1]

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INTRODUCTION

The ability of certain hydrocarbons and halogen-substituted hydrocarbons to inhibit the growth (as evidenced by a failure to gain in total body weight) of young rats ingesting a diet relatively low in sulfur-containing amino acids has been demonstrated. Among the compounds which have been reported to produce this effect are bromobenzene (*1*), naphthalene (*2*), anthracene (*3*). A similar effect has been obtained with iodoacetic acid (*4*) and cholic acid (*5*).

Recently this study has been extended to include the carcinogenic substances, benzpyrene and methylcholanthrene (*6*), and the non-carcinogenic compound, pyrene (*6*). The addition of *l*-cystine or *dl*-methionine to the diet containing the growth-retarding substance produced a prompt resumption of growth.

In every case the results apparently indicate that the inhibition of growth brought about by these substances is a result of imposing on the organism a new demand for the sulfur-containing amino acids in which the diet is restricted. As a result the organism has an insufficient amount of these essential amino acids for both detoxication and growth. Hence growth is able to proceed only after the more pressing need of detoxication has been met. The hypothesis that the sulfur-containing amino acids participate in the detoxication mechanism has been demonstrated most conclusively for bromobenzene (*1*), naphthalene (*2*), and anthracene (*3*) by the isolation from the urine of a conjugation product of the hydrocarbon and one of the sulfur-containing amino acids as a mercapturic acid.

The present investigation was undertaken to determine whether certain other compounds, especially the azo dyes and in particular butter yellow, which has been shown to produce primary liver tumors, would react in a similar manner to the above-mentioned compounds. Furthermore, this type of an investigation seemed to offer an opportunity to study various stages of liver-tumor formation under rigorous dietary control.

This report describes the results observed by the oral administration of *p*-dimethylaminoazobenzene (butter yellow) and the effect of supplementary sulfur-containing amino acids. The results on liver-tumor formation will be reported at a later date.

EXPERIMENTAL PROCEDURE

Twenty-one-day-old rats of Osborne-Mendel strain at the time of weaning were placed in individual metabolism cages where they were allowed to eat *ad libitum* a basal diet of the following composition: casein [2] 6 percent, cornstarch 50 percent, Crisco 24 percent, sucrose 15 percent, cod-

[1] *Presented before the thirty-third annual meeting of the American Association for Cancer Research, Pittsburgh, March 19 and 20, 1940.*

[2] *Furnished by the Casein Corporation of America.*

liver oil 1 percent, and salt mixture[3] 4 percent. In addition each animal received a daily supplement of 400 mg. of dried brewers' yeast. After the animals reached a weight of 75–85 gm., 60 mg. of butter yellow was added to each 100 gm. of diet.

A preliminary experiment indicated that this concentration of the dye would produce the desired retardation of growth without apparent toxic effect. The animals remained in relatively good health during the entire course of the experiment.

After 30–50 days on this diet, during which time the growth inhibiting effect of the dye was established, the supplement to be studied was incorporated into the butter-yellow-containing diet. The compounds studied were *l*-cystine, *dl*-methionine, taurine, cysteic acid, anhydrous sodium sulfate, and glycine. Each was added in the ratio of 500 mg. to 100 gm. of diet. The *l*-cystine, taurine, and glycine were obtained from the Eastman Kodak Co., and the *dl*-methionine was obtained from the Organic Chemical Manufacturers Division, University of Illinois. The cysteic acid was prepared by the oxidation of cystine with bromine by the method of Gortner and Hoffman (*8*). The anhydrous sodium sulfate was of the analytical reagent quality of the Mallinckrodt Co.

The changes in weight of the animals and the food consumption were recorded twice weekly throughout the course of the investigation.

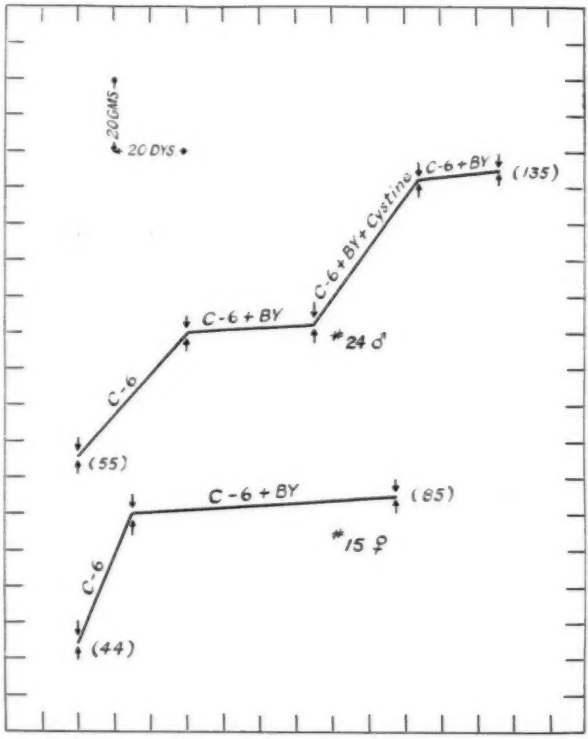

FIGURE 1.—Growth of rats on basal diet, and on basal diet with added butter yellow as influenced by various supplements. The diet employed in any portion of an experiment is indicated between two downward arrows representing the beginning and end of a period. The quantities of the supplements fed are given in the text. The meaning of the symbols C–6 and BY are explained in table 1. The initial and final body weights are shown in parentheses. Animals Nos. 24 and 15 are typical for these experiments and represent 61 and 80 animals, respectively.

[3] *Modified Osborne-Mendel salt mixture* (*7*).

RESULTS

Table 1 presents a summary of the average daily weight changes and food consumption under the different dietary regimes. Figures 1, 2, and 3 illustrate the growth rates of the animals. No animal died because of the toxic nature of the butter yellow. This is not in accord with the findings of the Japanese investigators, who found a high mortality due to the toxicity of the butter yellow. It must be pointed out, however, that the strain of rats and the basal diet employed by us was quite different from those used by Kinosita (*9*) and his group.

The addition of *l*-cystine or *dl*-methionine to the basal diet which contained the butter yellow resulted in a marked resumption of growth. These observations are similar to those obtained when methylcholanthrene and benzpyrene (*6*) were fed to rats under comparable conditions.

The failure of sodium sulfate, taurine, or cysteic acid to stimulate growth illustrated that the probable detoxication is not a general property of sulfate or oxidized sulfur but appears under the conditions of this experiment to be dependent upon the specific chemical requirement of the organism for cystine or methionine or substances giving rise to either of the latter in metabolism. Bennett (*10*) has shown that *l*-cystine disulfoxide, an intermediary oxidation product of cystine, when replacing *l*-cystine in the diet, could produce normal growth. The failure of glycine (which has been

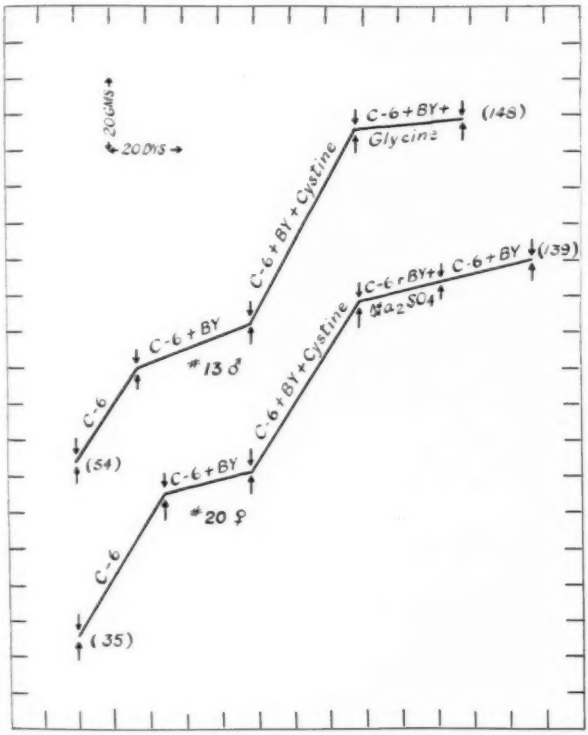

FIGURE 2.—Growth of rats on basal diet, and on basal diet with added butter yellow as influenced by various supplements. Designations for diets employed and body weights are the same as in figure 1. Animals Nos. 13 and 20 are typical for these experiments and represent 21 and 20 animals, respectively.

shown to detoxify benzoic acid) to stimulate growth under similar conditions would seem to indicate further that the detoxication is directly related to available organic sulfur in the form of *l*-cystine or *dl*-methionine or substances giving rise to these compounds.

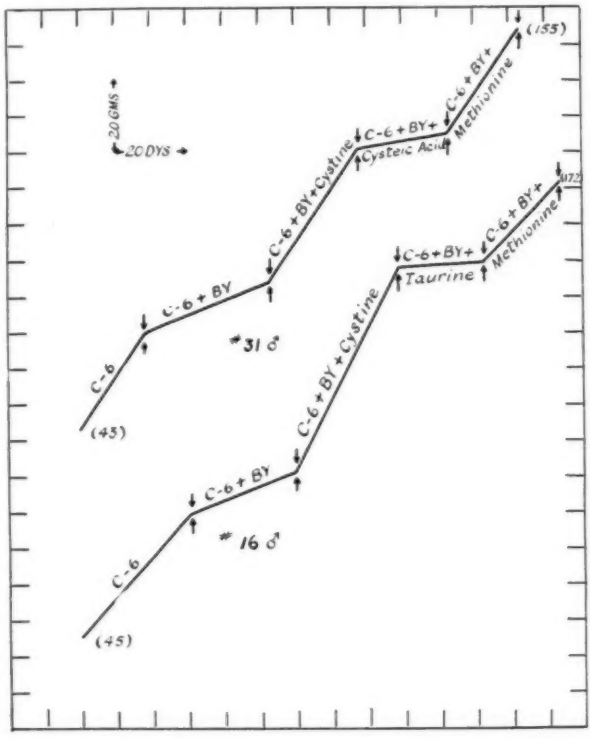

FIGURE 3.—Growth of rats on basal diet, and on basal diet with added butter yellow as influenced by various supplements. Designations for diets employed and body weights are the same as in figure 1. Animals Nos. 31 and 16 are typical for these experiments and represent 18 and 14 animals, respectively.

TABLE 1.—*Summary of average weight changes and average food consumption of animals receiving basal diet and various types of supplements*

Diet	Designation of diet in figures 1-3	Number of animals	Average daily weight change	Average daily food consumption
			Grams	*Grams*
Basal	C-6	107	+1.6	7.5
Basal + butter yellow	C-6 + BY	81	+.3	5.9
Basal + butter yellow + cystine	.....	77	+1.5	7.7
Basal + butter yellow + glycine	.....	21	+.5	7.3
Basal + butter yellow + sodium sulfate	.....	20	+.5	7.1
Basal + butter yellow + methionine	.....	16	+1.4	8.0
Basal + butter yellow + taurine	.....	14	+.4	6.7
Basal + butter yellow + cysteic acid	.....	18	+.5	7.1

The data presented suggest that the inhibition of growth of young rats on low-protein diets by butter yellow and certain toxic compounds is probably due to the utilization by the organism of some of the growth-essential protein constituents for detoxication rather than for the synthesis of new protein. In animal economy, detoxication takes precedence over synthesis. Thus the somewhat limited amount of cystine and methionine in the basal diet containing the butter yellow was needed for detoxication, and little

remained for the formation of new tissue. However, when additional cystine or methionine was added, sufficient amounts of these amino acids were available for both detoxication and growth.

SUMMARY AND CONCLUSIONS

The addition of dimethylaminoazobenzene to a basal diet of relatively low protein content produced a retardation of growth (failure to gain weight normally) when administered to young rats.

The addition of *l*-cystine or *dl*-methionine to the basal diet still containing the butter yellow resulted in a prompt stimulation in growth which was comparable with that observed in animals ingesting the basal diet alone.

The addition of taurine, cysteic acid, or sodium sulfate to the basal diet containing the butter yellow failed to stimulate growth. This seems to indicate that sulfur in the form of cystine, methionine, or substances giving rise to cystine or methionine are capable of functioning as detoxifying agents.

It is suggested that butter yellow probably produces a specific demand on the animal for a sulfur-containing amino acid, cystine or methionine, which is needed for detoxication. As a result, the synthesis of new tissue protein becomes limited. The addition of cystine or methionine appears to function as a part in the detoxication mechanism by supplying the necessary sulfur-containing amino acids for the synthesis of tissue protein.

Acknowledgment is hereby made to Majic S. Potsaid for technical assistance rendered.

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281167—41——5

Effect of Carcinogens on Small Free-Living Organisms

II. Survival Value of Methylcholanthrene-adapted Paramecium

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In the first paper of this series (1) we reported that after long adaptation of *Eberthella typhi* to methylcholanthrene, and to the gamma rays of radium, no structural, functional, or immunological changes were observed. Although these experiments, as far as they were carried, gave essentially negative results, observations on paramecia similarly adapted to carcinogenic agents have yielded very interesting data. This paper reports the increased survival period under conditions of inadequate food supply of certain individuals of a race of *Paramecium multimicronucleatum* which had been exposed to methylcholanthrene for more than a year.

MATERIALS AND METHODS

ADAPTATION TECHNIQUE

Our test organism was a strain of *Paramecium* obtained from a fresh water pond in nearby Maryland and subsequently identified as *P. multimicronucleatum* by Prof. T. M. Sonneborn, Department of Zoology, University of Indiana. All cultures were carried in 1-ounce specimen bottles with tightly fitting rubber stoppers to prevent evaporation. Each culture bottle contained 3 sterilized rice granules and 10 cc. of dilute paramecium medium prepared by adding 1 cc. of a stock solution [1] to 1,000 cc. of distilled water.

[1] *The stock solution consisted of the following: NaCl, 10.0 gm.; CaCl, 0.6 gm.; KCl, 0.4 gm.; and H_2O (distilled), 100.0 cc.*

Lately, we have buffered the paramecium medium with 1 cc. of a phosphate buffer (pH. 6.0) to each 100 cc. Our freshly distilled water was slightly alkaline in reaction. We found that the organisms thrive best by maintaining the medium well on the acid side. The optimum range apparently was from pH 6.0 to 6.4.

The medium having been prepared, 12 culture bottles were set up in series. To bottle No. 1, 0.1 cc. of a stock solution of methylcholanthrene in benzene (0.1 mg. per cubic centimeter) was added; to bottle No. 2, 0.2 cc. was added, and so on, bottle No. 10 receiving 1.0 cc., or 0.1 mg., of methylcholanthrene. After the benzene had completely evaporated from all the bottles, the 10 cc. of media and 3 rice grains were added, and all 12 bottles were inoculated with 0.5 cc. of a stock culture of *Paramecium*. After 10 days' growth at room temperature, organisms from each bottle were transferred to the corresponding bottle of a second series freshly prepared in the same manner.

As a control, adaptation of *Paramecium* to fluorescein was carried out in essentially the same manner. This dye being soluble, 0.1 cc. of a 1-percent solution was added to culture bottle No. 1. The other nine bottles in each series received graded amounts, up to 1 cc. in the tenth bottle. Adaptation tests on the effect of phenanthrene and 9-anthracene, using corresponding amounts of the chemicals as in

the methylcholanthrene tests, were also made. After a few transfers the organisms died out.

Thus all the organisms and all their descendants were exposed continuously to the methylcholanthrene. This test was started May 8, 1939, and at the present time (Oct. 1, 1940) has been transferred approximately every 10 days for 48 times, The exact number of cell divisions is, of course, not known. We believe the minimum to be more than 250 successive cell divisions.

TECHNIQUE OF SURVIVAL TEST

Pyrex glass depression plates (fig. 1) containing nine bowls of 1-cc. capacity each were used to observe the cell-division rate and the survival of normal organisms,

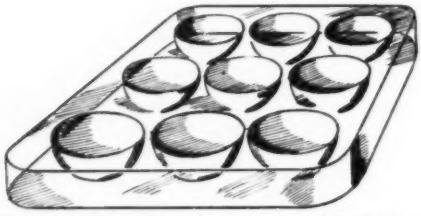

FIGURE 1. Depression plates for observing viability and multiplication of paramecia

of carcinogen-adapted organisms, and of organisms adapted to closely related non-carcinogenic hydrocarbons.

The depression plates were first placed in a concentrated chromate sulphuric acid solution for at least 24 hours; then they were rinsed in tap water and placed in 50 percent nitric acid for another 24 hours. Next they were placed in a liquid soap and cleaning solution prepared from a commercial powder known as Oakite (trisodium phosphate). After thorough rinsing, successively, in tap water, in distilled water overnight, and in the buffered paramecium medium they were drained and used immediately without drying.

About one-tenth cubic centimeter of the culture of organisms from the culture of *Paramecium* to be tested for survival was placed in a watch crystal containing at least 5 cc. of the plain salt-solution media. This diluted the organisms so that they could be easily picked up with a capillary pipette, one or two at a time, under a dissecting microscope and transferred to the bowls of the depression plates, each of which contained 1 cc. of the salt-solution medium. Routinely, 2, sometimes 4, organisms were placed in each bowl which gave 18 or 36 organisms to each plate. In any given series the number was the same in each bowl.

The population counts of each plate were recorded daily. Since this method of transferring the organisms from the cultures to the plates involved two dilution procedures, we believe the amount of methylcholanthrene that reached the individual bowls of the plates was negligible.

When our paramecia were first exposed to methylcholanthrene (May 1939), no effort was made to exclude ultraviolet light. All tests were performed in diffuse daylight until the organisms had been exposed continuously to the carcinogen from May 10, 1939, to April 11, 1940, for 32 consecutive transfers. Since the latter date, daylight has been excluded from the laboratory, and ceiling lights have been covered with five layers of a yellow cellophane (tango shade) to exclude ultraviolet radiations from such sources as far as possible. However, we have never observed any photosensitizing effect unless the organisms were deliberately exposed to sunlight.

It should be emphasized that *Paramecium* in our hands was found to be extremely sensitive to slight environmental changes. Results of a single test were never considered as conclusive. There

was a marked variability of the survival period even in various bowls of the same depression plate, especially when these tests were first performed. Lately with the careful cleaning and washing process, combined with a final soaking of the depression plates in the buffered medium, far more uniform results have been obtained.

RESULTS

More than 15 separate tests were carried out in which daily population counts were made in order to study the survival period of the methylcholanthrene-adapted organisms in contrast with normal unadapted controls and with organisms adapted to a non-carcinogenic dye. All of our survival tests were set up with the simple paramecia salt solution described above. In nearly every case the untreated organisms have shown some proliferation for 1 or 2 days, although there is no organic material present. After a few days, the autotrophic bacterial count undoubtedly increases.

Figure 2 shows the daily census of 1 representative lot of paramecia after they had been adapted to methylcholanthrene for 388 days (dotted line). During this interval the organisms had been transferred 35 times at 10- or 12-day intervals. The concentration of methylcholanthrene was 0.01 mg. per 10 cc. of media, or 1 gamma per cubic centimeter.

The daily population of untreated organisms from cultures of the same age (21 days old) as the adapted organisms is also recorded (solid line).

At the beginning of this test the populations of the adapted and of the control organisms were the same, namely, 288 organisms each. Eight depression plates, 9 bowls to a plate, and 4 organisms to each bowl were used.

A slight increase in population after 24 hours was noted in both groups—the adapted organisms increased from 288 to 322 while the controls increased from 288 to 310. After the second day both groups fell off in numbers, and by the ninth day all the controls were dead. The adapted organisms continued to die off at a slower rate, and on the eleventh day there was a secondary rise in population.

The test was discontinued on the fifteenth day because the bowls were rapidly drying up. However, there were 80 organisms still alive. Tests of this kind have been repeated 15 times. In every instance the

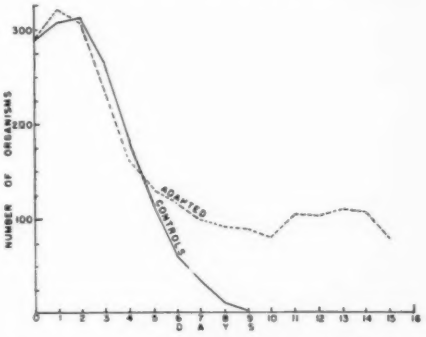

FIGURE 2.—Daily survival census of representative lot of a strain of *Paramecium* which had been adapted to methylcholanthrene for 388 days.

methylcholanthrene-adapted organisms have lived longer. As a rule, but not invariably, the population of the adapted organisms has reached greater numbers than the controls, and secondary rises in population have been very common among the adapted organisms.

In figure 3 is recorded the daily census of normal paramecia, of paramecia adapted to methylcholanthrene, and to a non-carcinogenic dye, fluorescein. The fluorescein-adapted organisms had been transferred 42 times in a concentration of 0.1 mg. fluorescein per cubic centimeter of medium. The adaptation process was started May 18, 1939, and the survival test recorded in figure 3 was set up September 5, 1940. The methylcholanthrene-adapt-

ed organisms came from the same series as those recorded in figure 2 and now had been transferred 44 times. Attempts to adapt paramecia to the non-carcinogenic hydrocarbon, phenanthrene, by using the same concentration as employed in the methylcholanthrene series (1 gamma per cubic centimeter) failed. This concentration proved to be toxic. The organisms did not survive. 9-Methylanthracene, another non-carcinogenic hydrocarbon, also proved toxic at the same concentration, but recently a series has been started in which the paramecia have survived by using only 0.1 gamma per cubic centimeter of 9-methylanthracene. However, adaptation to this chemical has not gone far enough to give conclusive results as to its influence on the organisms.

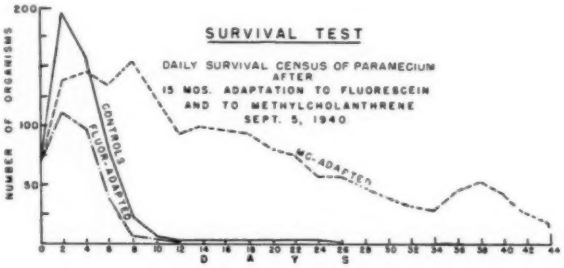
SURVIVAL TEST
DAILY SURVIVAL CENSUS OF PARAMECIUM AFTER 15 MOS. ADAPTATION TO FLUORESCEIN AND TO METHYLCHOLANTHRENE SEPT. 5, 1940
NUMBER OF ORGANISMS
200
100
50
CONTROLS
FLUOR-ADAPTED
MC-ADAPTED
0 2 4 6 8 10 12 14 16 18 A 20 22 Y 24 26 28 30 32 34 36 38 40 42 44
D
S

FIGURE 3.—Daily survival census of *Paramecium* after 15 months' adaptation to fluorescein and to methylcholanthrene, September 5, 1940.

It should be noted that the controls, the methylcholanthrene-adapted and the fluorescein-adapted organisms all revealed an increase in population for the first 2 or 3 days after which the fluorescein-adapted organisms and the controls steadily fell off. The fluorescein-adapted organisms were dead on the fourteenth day, and the controls on the twenty-fifth day. The methylcholanthrene-adapted organisms revealed at least three secondary increases in population—on the eighth, the fourteenth, and the thirty-sixth day. Eighteen organisms were still alive on the forty-fourth day when the individual bowls contained not more than 0.1 cc. of medium each. Thus these organisms were alive 18 days after all controls were dead and 32 days after the fluorescein-adapted organisms were dead.

DISCUSSION

A number of tests designed to study the immediate effect of methylcholanthrene on paramecia were made. Stimulation of cell division was found as a rule in these latter experiments—results which accord with observations of the proliferative effect of carcinogens on bacteria as reported by Goldstein (*2*) and with those of Wolman (*3*) who observed increased cell division on *Paramecium* under the influence of three carcinogenic hydrocarbons. However, we do not believe that the increased proliferation of single-celled organisms is any index of carcinogenesis, as suggested by Goldstein, since we have observed the same effect with a non-carcinogenic dye, fluorescein, and with an amino acid, l-cystine, when employing these agents under the conditions of our survival technique. The mechanisms of these stimulation effects are not known. It is so prompt and pronounced, however, in the case of cystine-treated organisms as to suggest that the paramecia readily utilize this amino acid as food.

The immediate effect of methylcholanthrene on paramecia in proper concentration resulted usually (1 out of 7 tests) in a stimulation of cell division, but there is no evidence from any of our experiments

which would suggest an increase in the period of survival of individual organisms. Nor have we been able to determine just when during the adaptive process, the greater resistance to such starvation conditions takes place, since survival tests were not carried out until the methylcholanthrene-adapted organisms had been transferred 25 times. In fact, it was not until then that we noticed that many of the methylcholanthrene-exposed cultures remained alive long after all the corresponding controls were dead. It was this observation which led us to carry out the survival tests on individual organisms in depression plates.

At the present time no theory is advanced as to the mechanism of this "toughening" process. We have reason to believe, however, that it does not appear at the time of first exposure of the strain to the carcinogenic agent. Adaptation of the same strain to the gamma rays of radium have shown a decided increase in motility over the controls, but this visible phenomenon was not observed until the organisms had been exposed to the irradiation for 3 months and became apparent gradually from the eighth to the tenth transfer. This latter observation will be reported in detail in a later publication.

It is believed our tests show that methylcholanthrene and perhaps other chemical carcinogens in suitable amounts are not destructive to living paramecia. They appear, on the contrary, to enhance certain vital functions. Furthermore, their action appears to us to have a cumulative as well as an immediate effect. There seems to be a delayed response which is not manifested at once but which may become apparent in some subsequent generation.

One might be inclined to interpret these results to mean that the chromosomes of the cell or the cellular components of heredity are primarily affected by carcinogenic agents. These same carcinogenic agents do not induce cancers in experimental animals in a few days. The process of experimental carcinogenesis is notoriously slow. Hence, it is conceivable that carcinogenic agents may not transform a single normal cell immediately into a cancer cell. The cell and its descendants must be exposed to the carcinogen over a considerable number of cell divisions before carcinogenicity becomes finally established. Such a concept also fits nicely with the observation that cancer not infrequently arises at the site of chronic irritation. Once established, the cancer process is irreversible, but as yet we have not tested the permanency of the changes observed in our methylcholanthrene-adapted paramecia.

Furthermore, we believe that the data here recorded are of general biologic interest, especially to those investigators concerned with the aging process of individual organisms (gerontology). We are not aware of any instance in the entire field of biologic research where the average life span of individuals of any species has been increased.

While we believe that these observations are suggestive and that they open up a new and interesting method of approach to the study of the action of carcinogenic agents on living cells, we are not prepared at present to state that the technique of species adaptation described above affects favorably the inherent viability of the *Paramecium*.

We are now prepared, however, to contrast the behavior of our radium and methylcholanthrene - adapted organisms with that of the normal organisms under the influence of various growth-stimulating and growth-inhibiting biochemicals.

Finally, it should be emphasized that we make no claim that our carcinogen-

adapted free-living organisms are analogues of mammalian cancer cells induced by these same agents, but the results are suggesstive.

SUMMARY

1. Certain individual organisms from a strain of *Paramecium multimicronucleatum* exposed continuously to methylcholanthrene for more than 1 year through numerous cell divisions possess greater survival value than do organisms from untreated cultures of the same strain and age.

2. The same strain of organisms adapted to a non-carcinogenic dye did not survive as long as untreated organisms.

3. The technique of strain adaptation and the method of testing survival value are described.

4. The biologic significance of these observations and their possible relationship to the mechanism of carcinogenesis are discussed.

ACKNOWLEDGMENT.—In carrying out this work the authors wish to acknowledge many helpful suggestions from various members of the staff of the National Cancer Institute.

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Tumors in Mice Injected With Colloidal Thorium Dioxide

By H. B. ANDERVONT, *senior biologist*, and M. B. SHIMKIN, *assistant surgeon, National Cancer Institute, National Institute of Health, United States Public Health Service*

In 1933 Oberling and Guérin (*1*) reported the production of sarcomas in rats following the intraperitoneal injection of colloidal thorium dioxide. Several investigators (*2–5*) produced sarcomas at the site of subcutaneous injection of the compound in rats, and subcutaneous tumors were also induced in mice (*3, 5*). Of nine such tumors in mice, arising 39 to 94 weeks after an injection of 0.2 cc. of the agent, Selbie (*3*) diagnosed six as spindle-cell sarcomas and three as osteosarcoma, histiocytoma, and capillary angio-endothelioma, respectively. Foulds (*6*) obtained one carcinoma and three sarcomas in nine female guinea pigs 2.5 to 3 years after four monthly injections of 0.2 to 0.3 cc. of the compound into the base of a nipple. Malignant neoplasms have not been reported in man, although granulomatous lesions following intramammary injection of colloidal thorium dioxide have been observed (*7*).

It was briefly noted elsewhere (*8*) that subcutaneous neoplasms in mice following the injection of colloidal thorium dioxide were obtained in this laboratory, but that primary pulmonary tumors could not be induced. In the present paper the experiment is presented in full.

EXPERIMENTAL PROCEDURE

Mice of strain A, obtained from the Roscoe B. Jackson Memorial Laboratory, and male mice of strain C_3H, raised in this laboratory, were used. The animals were maintained under similar environmental conditions, on a diet of Purina dog chow and an unlimited supply of water. The mice were 3 months of age at the time of injection.

Ten C_3H males and ten strain A females were injected subcutaneously in the right axilla with 0.2 cc. of colloidal thorium dioxide.[1] Ten strain A females received the same dose intraperitoneally. Of the 30 animals, 3 strain A mice died during the first few months and are not included in these results. Mice injected intraperitoneally were killed 9 and 12 months after injection, and the subcutaneously injected mice were permitted to live until 18 months after the experimental procedure.

It was found that 0.3 cc. or more of the preparation given intravenously killed the mice within 24 hours. Repeated weekly injections of 0.2 cc., however, were tolerated. Eighteen female strain A mice received a single injection of 0.2 cc. intravenously. In order to ascertain the effect of larger doses of thorium dioxide on the pulmonary tissue of mice susceptible to tumors of the lung, 10 strain A males received 4 weekly doses of 0.2 cc., or a total of 0.8 cc. Of these 2 died within 48 hours after the last injection. Another group of 20 strain A males were injected intravenously 3 times, at weekly intervals, with 0.2 cc., or a total of 0.6 cc. None of these animals died. The mice were killed at intervals from 3 to 14 months after injection.

RESULTS

As presented in table 1, 35 strain A mice that received 0.2 to 0.8 cc. of colloidal

[1] *Thorotrast, stated to contain 24 to 26 percent of ThO_2 by volume. We are indebted to the Heyden Chemical Corporation for the preparation, supplied through Dr. Egon Lorenz.*

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TABLE 1.—*Tumors in mice injected with colloidal thorium dioxide*

[Numerator=number with lung tumors; denominator=number of mice killed]

Strain of mouse	Number of mice	Route of injection	Dose	Pulmonary tumors							Subcutaneous tumors		
				Months after injection							Effective total	Months after injection	
				3	4	5	6	9	12	13–18		15	18
			Cc.										
C_3H	10	Subcutaneous	0.2							1/10	5	1	
A	9	do	.2			0/2		0/2		2/5	4	1	1
A	9	Intraperitoneal	.2					2/4	2/5				
A	18	Intravenous	.2	0/4			0/3	2/3	1/4	3/4			
A	8	do	[1].8		0/8								
A	20	do	[2].6			1/10	2/10						
Incidence of spontaneous lung tumors in A mice at corresponding ages (*11*) percent				9	15	17	34			72			

[1] Administered in 4 weekly doses.

[2] Administered in 3 weekly doses.

thorium dioxide intravenously were killed 3 to 6 months after injection. Ten strain A mice were permitted to live from 9 to 14 months after the experimental procedure. In none of these groups, or in the mice injected subcutaneously or intraperitoneally, was the incidence of primary pulmonary tumors increased above the spontaneous occurrence. None of the mice had multiple pulmonary tumors, which is further evidence of the negative effect of the compound on the lungs of these mice (*8*).

The lungs of the mice injected intravenously were seen to contain particles of thorium dioxide throughout the course of the experiment. The reaction evoked was of the same nonspecific type as that seen after the intravenous administration of various ore and quartz suspensions: perivascular and subpleural lymphocytic nests, with slight thickening of the alveolar walls (*9*). The thorium dioxide particles were visible also in the liver and in the spleen. The latter organ was uniformly enlarged and mottled and displayed a productive fibrous splenitis. The splenic architecture was markedly altered. The follicles were obliterated, the sinuses were dilated, and throughout the organ there were areas composed of large pale cells resembling epithelioid cells. These lesions showed fibrosis and occasional necrosis in the central regions. In other portions of the spleen there were numerous pigment-laden phagocytes.

Three subcutaneous tumors were induced in mice which received the agent subcutaneously. The first two appeared at 15 months, one in a C_3H male and the other in an A female. The third tumor was found in a strain A mouse 18 months after injection. At the time of appearance of the first tumor five C_3H males and four strain A females were alive, so that the three tumors were obtained from an effective total of nine mice.

The two tumors which appeared in the A mice were spindle-cell sarcomas, measuring 16 and 18 mm. in diameter after progressive growth of several weeks (figs. 1 and 2). The tumors were of the same histologic type as the spindle-cell sarcomas seen after injections of carcinogenic hydrocarbons, except that they contained thorium dioxide particles, many of which were phagocytized by macrophages. The tumor which arose in the C_3H male mouse was a hemorrhagic mass measuring 16 mm. in diameter. It con-

sisted of cavernous spaces filled with blood and separated by thin walls of connective tissue lined by flattened cells resembling endothelium (fig. 3). It was diagnosed as a hemangioma, and resembled the tumor designated as a capillary angio-endothelioma by Selbie (*3*). No attempts were made to transplant these tumors.

The 10 C_3H males, killed 13 to 18 months following the injection, had 4 hepatomas, an incidence encountered spontaneously in this strain (*10*).

DISCUSSION

Since the carcinogenicity of colloidal thorium dioxide for the subcutaneous tissue of mice has been established (*3, 5*), the primary object of this experiment was to ascertain whether the agent would also elicit pulmonary tumors in strain A mice.

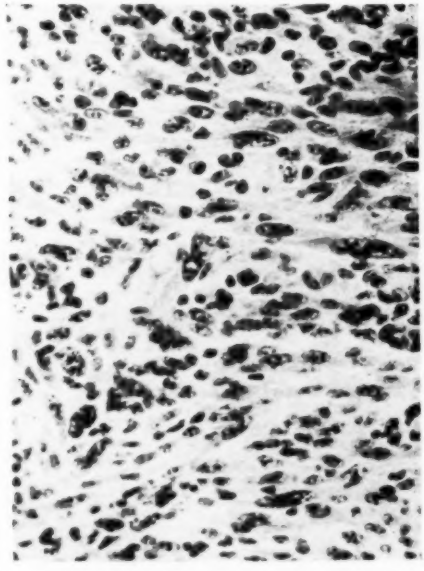

FIGURE 1.—Spindle-cell sarcoma in female strain A mouse, 15 months after subcutaneous injection of 0.2 cc. of colloidal thorium dioxide. Haematoxylin and eosin. × 355.

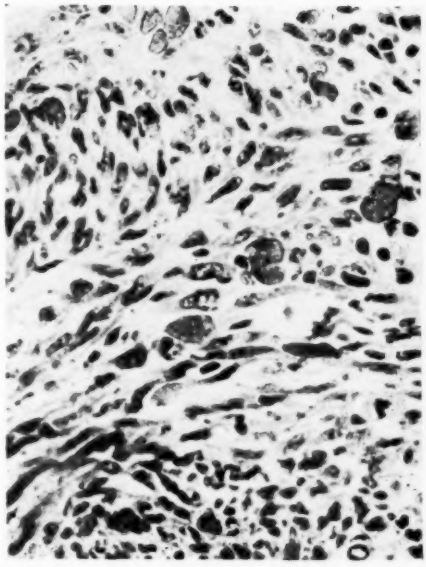

FIGURE 2.—Spindle-cell sarcoma in female strain A mouse, 18 months after subcutaneous injection of 0.2 cc. of colloidal thorium dioxide. Haematoxylin and eosin. × 415.

With carcinogens tested previously (*8*), it was found that primary pulmonary tumors appeared in strain A mice within 3 months after intravenous injection, regardless of the latent period for the subcutaneous tissues. The incidence of the pulmonary tumors in the group and the multiplicity of the tumors per individual animal could be used as an index of the carcinogenic potency of the compounds. The pulmonary neoplastic reaction apparently could be produced with smaller doses than the subcutaneous sarcomas.

The data with colloidal thorium dioxide, extending over a year after intravenous injection and with doses three to four times the dose which produced subcutaneous tumors, are sufficient to conclude that primary pulmonary tumors were not induced with this agent in strain A mice.

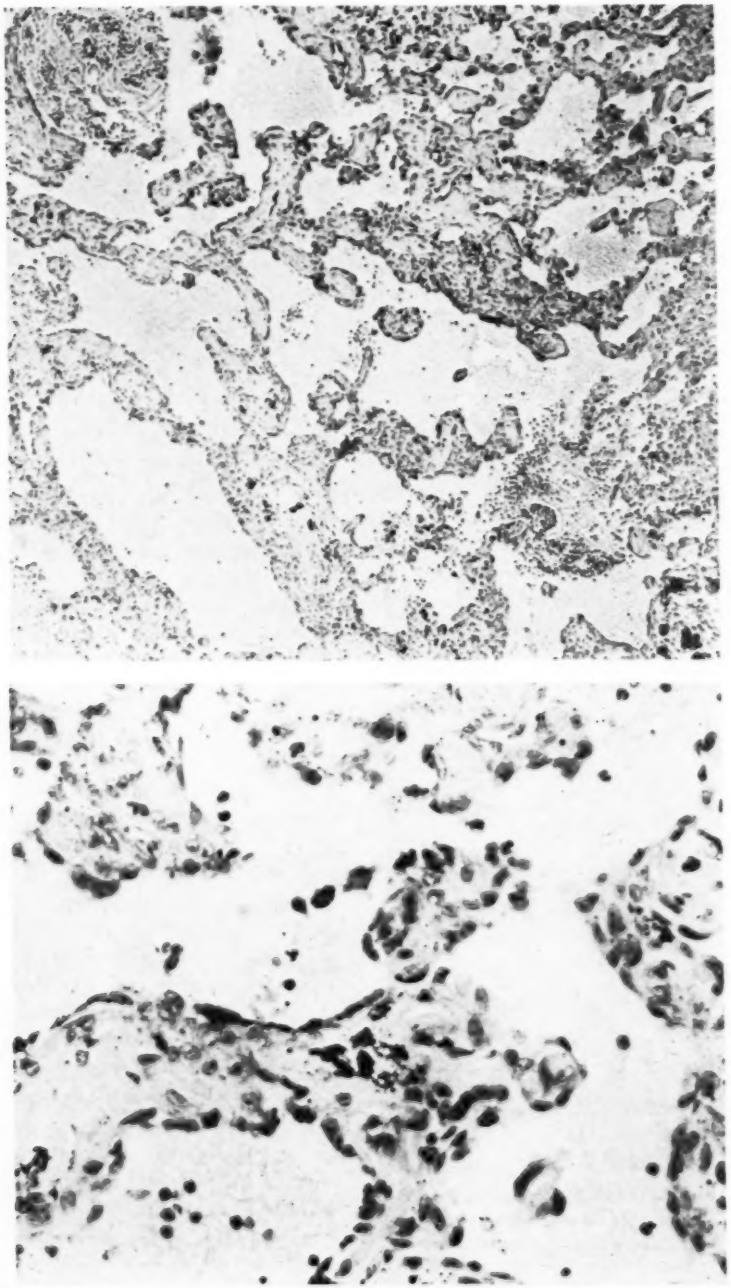

FIGURE 3.—Hemangioma in male mouse of strain C_3H, 15 months after subcutaneous injection of 0.2 cc. of colloidal thorium dioxide. Haematoxylin and eosin. *A*, × 95; *B*, × 415.

CONCLUSION

Three tumors, two spindle-cell sarcomas and one hemangioma, were induced in nine mice 15 to 18 months following the subcutaneous injection of 0.2 cc. of colloidal thorium dioxide. The compound did not induce primary pulmonary tumors in strain A mice following the intravenous injection of 0.2 to 0.8 cc.

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Preparations of Dispersions of Carcinogenic Hydrocarbons and Hormones With the Aid of Dioctyl Ester of Sodium Sulfosuccinate (Aerosol O. T.)

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Most hormones and carcinogenic hydrocarbons are soluble only in fats and fat solvents. For some purposes, such as intravenous injection, it is desirable to have aqueous dispersions of these chemicals. Several methods of preparing dispersions of carcinogenic hydrocarbons have been described, but aqueous dispersions of hormones have not been reported.

Three methods of preparing aqueous dispersions of carcinogenic hydrocarbons are found in the literature. Berenblum (*1*) used the following procedure:

> 10 cc. of a 0.1 per cent. solution of this compound [1,2,5,6-dibenzanthracene] in pyridine is added drop by drop from a burette to 90 c. cm. of distilled water, the mixture being stirred throughout the process. The opalescent solution, thus produced, is then poured into collodion sacs and dialysed against water for two or three days until all the pyridine has been removed.

The concentration was increased to 0.4 mg. per cubic centimeter by adding 20 cc. of a 0.2-percent solution of 1,2,5,6-dibenzanthracene [1] in pyridine to 80 cc. of a 6-percent solution of gum acacia.

Boyland (*2*) dissolved 0.3 gm. of purified dibenzanthracene in 100 cc. of acetone. One part of the acetone solution was poured into 10 parts of a warm gelatine solution in distilled water (0.5 percent), and the mixture heated until all the acetone was removed. This dispersion contained 0.3 mg. of the hydrocarbon per cubic centimeter.

Lorenz and Andervont (*3*) prepared dispersions of dibenzanthracene and of 20-methylcholanthrene [2] in horse serum saturated with cholesterol. An excess of a concentrated solution of cholesterol in ether was added to horse serum. The mixture was shaken for several hours, the ether blown off at room temperature, and the excess cholesterol removed by filtration. An appropriate amount of the chemically pure carcinogenic hydrocarbon dissolved in ether was then added, the mixture shaken for 4 hours, and the ether blown off at a temperature of about 0° C. Homogenous suspensions containing 0.1 mg. of dibenzanthracene or methylcholanthrene, suitable for intravenous injection, were obtained. The method was improved (*4*) so that concentrations of 1 mg. per cubic centimeter were reached, and more recently [3] suspensions containing 20 mg. of the hydrocarbon per cubic centimeter of serum have been prepared.

These dispersions were found active in producing tumors in experimental animals following subcutaneous, intramuscular, intraperitoneal, and intravenous injection (*4*, *5*, *6*).

The following investigation, initiated in

[1] *Hereinafter referred to as dibenzanthracene.*

[2] *Hereinafter referred to as methylcholanthrene.*

[3] *Lorenz, E.: Unpublished data.*

April 1939, describes a new method of preparation of dispersions of carcinogenic hydrocarbons. The technique is applicable also to estrogens and androgens, and recently a similar dispersion of desoxycorticosterone acetate has also been made.

EXPERIMENTAL PROCEDURE

PREPARATION OF DISPERSIONS

When aqueous dispersions are prepared by the addition of ether, alcohol, or acetone solutions of carcinogenic hycrocarbons, it is noted that flocculation often occurs after the solvent has been boiled or blown off, especially with concentrations above a few tenths of a milligram per cubic centimeter. Some of the material adheres to the glass or collects near the surface of the water. Addition of a protective colloid prevents this only to a limited degree.

It was found that the addition of a suitable wetting agent prevents both the flocculation and the adherence to the glass. It is therefore assumed that the flocculation is due to insufficient wetting of the suspended particles by the surrounding liquid. The wetting power of a chemical is dependent upon its ability to reduce the surface tension of the solution and the interfacial tension between the solid and the liquid. A large number of wetting agents are known and used commercially. In these experiments, use was made of the dioctyl ester of sodium sulfosuccinate [4] (7). According to the manufacturer it is practically chemically pure. Its hydrogen-ion concentration in aqueous solution is between 6.5 and 7. It is stable in acid and in weak alkali ($pH \leqq 9$).

Two methods of preparation of dispersions were devised. One is suitable for the production of dispersions for subcutaneous or intraperitoneal injection, and the other is used in making dispersions which can be administered intravenously.

[4] *Aerosol O. T., American Cyanamid Co., Stamford, Conn. Throughout the paper, Aerosol is used to describe the chemical.*

DISPERSIONS FOR SUBCUTANEOUS INJECTION

To the crystalline product is added a small amount of a 5-percent solution of Aerosol in water. The mixture is ground in a mortar, thus breaking up the crystals into a fine paste. It is important that the grinding of the crystals be done with Aerosol of a concentration between 1 and 5 percent. If lower concentrations of the wetting agent are desired, water is added to the paste afterwards.

The particles stay in suspension if the final concentration of Aerosol is approximately 5 percent. With lower concentrations the particles settle out on standing, but can be resuspended easily by shaking.

The dispersions given in table 1, which were sufficiently fine to pass through a 28-gauge needle, were prepared according to this method.

TABLE 1.—*Concentration of various compounds and of Aerosol in dispersions used for subcutaneous injection*

Compound	Concentration of compound per cubic centimeter	Concentration of Aerosol
	Milligrams	*Percent*
20-Methylcholanthrene	2.5	5
3,4-Benzpyrene	2.5	5
1,2,5,6-Dibenzanthracene	2.5	5
Estrone	1.0	.1

DISPERSIONS FOR INTRAVENOUS INJECTION

In dispersions for intravenous injection, a colloidal solution of cholesterol in distilled water is first prepared. To 1 liter of distilled water is added 100 cc. of ether containing approximately 2 gm. of cholesterol. The ether is blown off, at first at a temperature a few degrees above 0° C., and finally at room temperature. After filtering, a stable colloidal suspension of cholesterol in water is obtained. This suspension will tolerate boiling.

TABLE 2.—*Concentration of various compounds and of Aerosol in dispersions used for intravenous injection*

Compound	Concentration of compound per cubic centimeter	Concentration of aerosol
	Milligrams	*Percent*
Estrone	1.0	0.033
Testosterone	3.0	.06
Do	5.0	.05
20-Methylcholanthrene	.2	.03

To the cholesterol colloid is added the chemical dissolved in ether. It is advisable to use a nonsaturated ether solution of the compound to be dispersed. The ether is then blown off as described in the preparation of the cholesterol dispersion.

Flocculation of the particles and adherence to the glass are observed at this stage. Aerosol is added to produce a concentration of 0.01 percent. The suspension is then shaken with glass beads for several hours. If flocculation is still present, more Aerosol is added. Usually a concentration of 0.02 to 0.05 percent of Aerosol is sufficient to produce a fine dispersion.

This procedure can be modified by dissolving the chemical to be dispersed in alcohol, adding the alcohol solution to the cholesterol colloid, and boiling off the alcohol. It is believed that the ether solution gives somewhat better results than the alcohol method.

Table 2 summarizes the dispersions prepared according to this method. The 0.5-percent testosterone dispersion was prepared by the alcohol technique.

If only a small amount of the chemical to be dispersed is available and higher concentrations are desired, it is advisable to prepare first a 0.1-percent dispersion (1 mg. per cubic centimeter). The preparation is then centrifuged until all the material is deposited at the bottom, and the supernatant fluid is decanted until a desired concentration is reached. The chemical is redispersed by shaking.

These dispersions will settle out on standing, but are easily brought into suspension by shaking. No change appears to take place in the preparations kept in the refrigerator for at least 6 months.

BIOLOGIC EVALUATION OF DISPERSIONS

TOXICITY OF AEROSOL

Table 3 presents the data on the toxicity of Aerosol in mice. Mice of strain A and C_3H and A backcross mice, weighing 25 to 30 gm. and about 3 months old, were used. It is seen that the animals tolerate 0.2 cc. of a 5-percent solution subcutaneously, 0.5 cc. of a 0.2-percent solution intraperitoneally, and 0.25 cc. of a 0.5-percent solution intravenously. There is apparently little, if any, cumulative general toxicity since the solutions can be given repeatedly.

Solid pellets of Aerosol, weighing 30 mg., were implanted subcutaneously into strain A backcross mice. Severe ulceration at the site of injection appeared within 1 week. None of the mice died, and grossly the ulcerated lesions healed within 3 weeks. At autopsy several weeks later the pellets

TABLE 3.—*Toxicity of Aerosol in mice*

Route	Concentration in water	Dose	Number of mice	Number that died
	Percent	*Cc.*		
Subcutaneous	10.0	0.5	3	3
	10.0	.25	3	2
	5.0	.2	15	0
	.5	[1] 7.5	5	0
Intraperitoneal	1.0	.25	6	6
	.2	.5	11	0
	.1	.25	5	0
	.1	[2] 5.0	5	0
Intravenous	1.0	.25	5	5
	.5	.5	5	5
	.5	.25	25	1
	.1	.5	17	0
	.5	[3] .75	3	0
Oral	.1	[4] 800	8	0

[1] 15 doses, every other day.
[2] 10 doses, weekly.
[3] 3 doses, weekly.
[4] 4 cc. per day for 7+ months.

were not found, and the lesion at the site of injection appeared healed histologically.

Strain A mice injected subcutaneously with 0.2 cc. of a 5-percent Aerosol solution were killed at various periods, from 3 to 120 days, after the administration. An acute ulcerative lesion developed at the site of injection. The area showed marked edema, congestion, and infiltration of polymorphonuclear leukocytes, mononuclear cells, and lymphocytes. There was extensive degeneration and necrosis of the voluntary muscle fibers in the region of the injected Aerosol, which was represented by irregularly sized vacuoles in the tissue. Regeneration of muscle and epidermis began early and was practically complete by 4 weeks, by which time the ulcers appeared to be well healed. In the animals examined later, the skin over the injection site showed fibrosis and loss of hair follicles. There was perineural and periosteal fibrosis of the deeper tissues; the musculature was apparently restored to normal. The character of the inflammatory lesion due to Aerosol was similar to that produced by a single subcutaneous injection of 0.05 cc. of oil of turpentine, except that the latter was more intense and showed more liquefaction.

In the animals injected with Aerosol, the liver in the early stages showed rather marked increase in mitotic figures in the absence of any demonstrable liver damage. There was a moderate degree of myelopoiesis of the spleen during the first 10 days of the experiment.

With intravenous injection of doses above 0.25 cc. of a 0.5-percent Aerosol solution in water, there was a sudden collapse and death in most of the animals. In the surviving mice, the urine became chocolate-colored within a half hour, evidence of hematuria due to severe hemolysis. The escape of some of the 0.5-percent solution into the perivenous tissues of the tail upon intravenous injection also caused ulceration and loss of the appendage in some animals of strain A.

With fatal intravenous doses, the mice showed congestion and petechiae in the lungs. With doses of 0.5 cc. of a 0.1-percent solution, no pathologic changes were observed.

There were no consistent or significant gross or microscopic lesions in the mice receiving Aerosol orally.

ACTIVITY OF DISPERSIONS OF CARCINOGENIC HYDROCARBONS

The dispersions of methylcholanthrene, 3,4-benzpyrene and dibenzanthracene in 5-percent Aerosol (table 1) were injected subcutaneously in 0.2 and 0.1 cc. amounts into male mice of strain C_3H, so that each mouse received 0.5 or 0.25 mg. of the hydrocarbon. The mice were examined weekly for the presence of a progressively growing solid mass at the site of injection. None of the mice of this strain developed ulceration at the site of the injection.

Table 4 summarizes the findings. Microscopic examination of representative tumors in these animals revealed them to be sarcomas like those usually obtained with these carcinogenic hydrocarbons.

In previous experiments with subcutaneously injected hydrocarbons dispersed in cholesterol-saturated horse serum, tumors were obtained in 27 out of 30 C_3H males which received 1 mg. of dibenzanthracene (average latent period, 24.7 weeks) (*4*), and in 11 out of 13 C_3H males injected with 1 mg. of methylcholanthrene (average latent period, 13.0 weeks).[5] The present results are in close agreement with these findings.

The 0.02-percent methylcholanthrene dispersion containing 0.03 percent of Aerosol (table 2) was tested for activity by intravenous injection into strain A mice.

[5] *Shimkin, M. B., and Lorenz, E.: Unpublished data.*

TABLE 4.—*Induction of subcutaneous sarcoma in C_3H male mice with 3 hydrocarbons dispersed in 5-percent aerosol*

Hydrocarbon	Dose	Number of mice	Weeks after injection										Number of tumors	Average latent time (in weeks)
			10	12	14	16	18	20	22	24	26–32	33–53		
	Milligrams													
Controls		10											0	
20-Methylcholanthrene	0.5	10	1	3	2			1			1		8	14.2
Do	.25	10		4	2		1		1				8	14.0
3,4-Benzpyrene	.5	10		4							2		6	17.8
Do	.25	10			1	2			2				5	17.4
1, 2, 5, 6-Dibenzanthracene	.5	10				1	1	2	3		2		9	21.5
Do	.25	10			1	2	2		1	1			7	18.0

Twenty strain A males and females, 3 months old, received a single intravenous injection of 0.5 cc., containing 0.1 mg. of the hydrocarbon. The mice were killed 8 weeks later and their lungs examined for the presence and the number of pulmonary nodules (*8*).

Of the 20 mice, 17 had pulmonary tumors, and the average number of tumors per positive animal was 3.1. This checks with the previous determination of the carcinogenic potency of 0.1 mg. of methylcholanthrene dispersed in horse serum, administered intravenously (*8*).

Twenty strain A mice, 3 months old, were injected intravenously with 0.5 cc. of cholesterol dispersion containing 0.03 percent Aerosol, as controls. Half of the mice were killed 20 weeks, and half 26 weeks after the administration. There were no pulmonary tumors in the mice killed at 20 weeks, and 3 single lung nodules in the 10 mice killed at 6 months. The incidence of spontaneous pulmonary tumors in strain A mice 9 months of age is over 30 percent (*8*).

The above results indicate that Aerosol is not carcinogenic within the limits of these experimental procedures, and that the presence of the compound with the resultant reduction in the surface tension in the tissue into which it is introduced does not influence the carcinogenic potency of the carcinogenic hydrocarbons.

The influence of Aerosol upon the growth of established tumors also was studied. Repeated intraperitoneal, subcutaneous, and oral administrations of aqueous solutions of aerosol were given to strain A backcross mice bearing transplanted sarcoma 37 and to strain C_3H females with spontaneous mammary carcinomas. There were no regressions or any observable effects on the growth of the tumors, or on the longevity of the mice bearing the tumors as compared with untreated controls.

ACTIVITY OF DISPERSIONS OF HORMONES

The dispersion of estrone, 1 mg. per cubic centimeter with 0.033 percent of Aerosol (table 2), was bio-assayed by Dr. I. T. Nathanson,[6] who found that the activity of the dispersion by the mouse test was approximately equal to the activity of an equivalent dose of estrone in oil.

The testosterone [7] dispersion, 3 mg. per 1 cc. with 0.06 percent of Aerosol, was assayed on 8-weeks-old capons and compared with the response to testosterone propionate in sesame oil. The compounds were given intramuscularly, either as single injections of 0.5 mg., or in five daily injections of 0.1 mg. The average increase in the height of the comb with the testosterone colloid, given either repeatedly or in a single injection, and with a single injection of testosterone propionate was 3 mm.;

[6] *Nathanson, I. T.: Personal communication.*

[7] *We are indebted to Dr. Gregory Stragnell of the Schering Corporation, Bloomfield, N. J., for supplying the testosterone.*

with repeated administrations of testosterone propionate in oil the combs increased an average of 5.5 mm. in height.

Strain A mice, weighing 25 to 28 gm., tolerated 0.2 cc. of the 0.3-percent testosterone dispersion and 0.25 cc. of the 0.1-percent estrone dispersion intravenously. The injections were repeated after 1 month. The mice were killed 20 and 26 weeks after injection. There was no increase in the incidence or the number of pulmonary tumors as compared with untreated controls.

CONCLUSION

A new method of preparation of aqueous dispersions of carcinogenic hydrocarbons and hormones, with the aid of dioctyl ester of sodium sulfosuccinate (Aerosol O. T.) is described.

The dispersions prepared by this method are stable and physiologically active. They are applicable for subcutaneous and intravenous injection into experimental animals.

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Effect of Colchicine and Bacterial Products on Transplantable and Spontaneous Tumors in Mice

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Interest in the effect of colchicine on tumors was aroused by a report (*1*) that Boyland noted profuse hemorrhage within the tumors of animals injected with the compound. Later Boyland and Boyland (*2*) published details of this work. The reader is referred to papers by Lits, Kirschbaum, and Strong (*3*) and by Brues, Marble, and Jackson (*4*) who presented excellent summaries of this subject together with much new material.

A series of experiments was performed in 1937 in which colchicine was injected into mice bearing transplantable or spontaneous tumors. It had been shown (*5*) that the administration of bacterial products to mice bearing propagable or induced sarcomas elicited a hemorrhagic reaction within, and in many instances complete regression of, the grafted tumors. The objects of the work reported here were (1) to ascertain whether colchicine was more effective than bacterial products in eliciting hemorrhage within, and regression of transplantable tumors; (2) whether repeated injections of colchicine influenced the growth of spontaneous mammary tumors in mice, and (3) whether colchicine and bacterial products supplemented each other in causing hemorrhage in transplantable tumors and, if so, whether the combined action of the two substances influenced the growth of spontaneous mammary tumors in mice. It was known (*5*) that treatment of mice bearing spontaneous mammary cancer with filtrate did not influence materially the growth rate of the tumors.

MATERIALS AND METHODS

Three-months-old mice of the inbred L (leaden) and A strains and albino backcross (ABC) mice, procured from the Roscoe B. Jackson Memorial Laboratory, were used in investigations on transplantable tumors. Strain C_3H mice, raised in this laboratory, were employed when tests were made on spontaneous mammary tumors. All experimental animals were kept under similar environmental conditions, on a diet of Purina dog-chow pellets and an unlimited supply of water.

Two transplantable tumors were used. One was the well-known sarcoma 37 of mice. The other was carcinoma F which arose in the lungs of a strain A mouse (*6*, *7*) and is propagable in the subcutaneous tissue of strain A or ABC mice. Results on transplantable tumors were obtained with cutaneous growths. Previous work (*5*), in which the technique for cutaneous inoculation was described, showed that these growths were excellent test objects for the hemorrhage-producing ability of bacterial filtrates. All spontaneous tumors were mammary carcinoma occurring in strain C_3H mice (*8*).

Colchicine (Merck) solutions were prepared by dissolving 10 mg. of the powder in 20 cc. of sterile physiological saline. Dissolving the colchicine in a few drops of 95-percent ethyl alcohol prior to the addi-

tion of saline facilitated preparation of the solution. Colchicine solutions were stored in the cold (4° C.) when not in use and none was kept for longer than 1 week. Filtrates of *Bacillus coli* were prepared by centrifuging and filtering (Berkefeld N) week-old nutrient-broth cultures. Dilutions were made in sterile physiological saline. One lot of filtrate was used throughout the study. The filtrate was also kept in the cold when not in use. All injections of colchicine solutions or bacterial filtrate were made intraperitoneally.

RESULTS

Strain L and ABC mice bearing 6-day-old growths of sarcoma 37 were used to ascertain the effective dose of colchicine, that is, the amount which elicited hemorrhage in transplanted tumors without killing the experimental animals. It was found that 0.15 to 0.2 mg. caused hemorrhage but killed the majority of mice; 0.1 mg. caused hemorrhage, was toxic, but killed few mice; 0.075 mg. produced only a few hemorrhages, and 0.05 mg. none. Thus, it was found that the effective dose of 0.1 mg. colchicine was close to the lethal dose and that less than 0.1 mg. did not elicit hemorrhage in an appreciable number of tumors. This narrow range of potency of colchicine is in contrast with the wider range of filtrate, for filtrate evoked hemorrhage in sarcoma 37 and was not lethal in 0.5-cc. amounts, while 0.1 cc. of the undiluted to 0.2 cc. of a 1:10 dilution also caused hemorrhage but no symptoms of toxicity.

Experiments were performed to ascertain whether repeated injections of 0.1 mg. of colchicine would be more efficacious in producing regression of tumors than a single injection. In these experiments strain L mice bearing 6-day-old growths of sarcoma 37 were each injected with 0.1 mg. They were then divided into five groups, which received a second injection of 0.1 mg. 24, 48, 72, 96, or 120 hours after the first injection. Practically all mice which received the second injection after an interval of 24, 48, or 72 hours died, while the majority receiving the second injection after an interval of 96 or 120 hours survived.

A repetition of 0.1-mg. injection of colchicine 96 hours after an initial injection of the same amount did not cause more regressions of sarcoma 37 than a single injection of 0.1 mg. Furthermore, repeated injections of 0.1 mg. colchicine every 96 hours up to a total of 0.3 or 0.4 mg. did not influence materially the growth of spontaneous mammary tumors in C_3H mice.

Repeated injections of 0.1 mg. colchicine every 96 hours produced definite symptoms of toxicity in the majority of mice bearing spontaneous tumors. This raised the question whether better results would attend the use of smaller doses for repeated injections. Consequently, a group of experiments was performed in which strain L mice bearing 6-day-old growths of sarcoma 37 received repeated injections of 0.05 mg. colchicine. It was found that a single injection of 0.05 mg. failed to elicit hemorrhage, but a repetition of the same dose given 3, 6, 9, or even 24 hours after the initial injection produced hemorrhage. If the second injection was given 48, 72, or 96 hours after the first, hemorrhage did not occur.

The administration of two doses of 0.05 mg. colchicine was no more effective in producing hemorrhage or regression of sarcoma 37 than a single injection of 0.1 mg. Repeated daily injections of 0.05 mg. of the drug failed to influence the growth of spontaneous mammary tumors in strain C_3H mice.

After it was found that single or repeated injections of colchicine were no more effective than a single injection of filtrate in

producing hemorrhage in transplantable tumors, efforts were made to ascertain whether colchicine and filtrate supplemented one another in evoking hemorrhage in transplantable tumors. These studies were carried out with strain L mice which had been inoculated cutaneously with sarcoma 37. The results of the investigation revealed that the two substances supplemented one another. The manner in which this was demonstrated is presented in table 1, where the results of two experiments are summarized.

In table 1 it is seen that 0.2 cc. of 1:20 to 1:80 dilutions of filtrate alone, or 0.05 mg. colchicine alone, produced no hemorrhage in the tumors but when the two were combined 72 percent of the tumors became hemorrhagic. It is also seen that 0.2 cc. of 1:5 to 1:10 dilutions of filtrate alone evoked hemorrhage while 0.05 mg. colchicine alone did not, but when the two were combined the majority of mice died. This demonstrated the supplementary action of the two substances for both hemorrhage-producing power and toxic action. It should be noted that although 0.3 cc. of undiluted filtrate did not kill and 0.1 mg. colchicine killed only 3 of 12 mice, 0.2 cc. of a 1:5 dilution of filtrate plus 0.05 mg. colchicine killed 11 of 12 mice, and 0.2 cc. of a 1:10 dilution of filtrate plus 0.05 mg. colchicine killed 6 of 12 mice. This indicates that the combined action of the two substances was more toxic for mice than the action of either substance alone.

In the experiment described above the colchicine and filtrate were not mixed before injection but were injected separately at practically the same time. Subsequent investigations showed that the substances supplemented each other only if the interval between injections of colchicine and filtrate was less than 24 hours. It was also found that the combined action occurred regardless of which substance was administered first.

A group of investigations was undertaken to determine whether the supplementary action of colchicine and filtrate could be demonstrated by using carcinoma F as the test tumor. Experimental evidence up to that time had shown tumor F to be refractory to the hemorrhage-producing power of the filtrate but susceptible to that of 0.1 mg. colchicine. If an amount of colchicine which was less than the effective dose plus an injection of filtrate evoked hemorrhage in tumor F,

TABLE 1.—*Supplementary action of bacterial filtrate and colchicine. Both injected at same time intraperitoneally, in strain L mice bearing 6-day-old cutaneous sarcoma 37*

Filtrate							Colchicine						Filtrate and colchicine								
Amount	Dilution	Number of mice	Tumor hemorrhages			Mice dying	Amount	Number of mice	Tumor hemorrhages			Mice dying	Amounts			Number of mice	Tumor hemorrhages			Mice dying
			+[1]	±[2]	−[3]				+[1]	±[2]	−[3]		Filtrate	Dilution	Colchicine		+[1]	±[2]	−[3]	
Cc.						*No.*	*Mg.*					*No.*	*Cc.*		*Mg.*					*No.*
0.3		5	5																	
.2		12	12																	
.2	1–5	12	11		1		0.05	5			5		0.2	1–5	0.05	12	12			11
.2	1–10	12	6		6		.05	5		1	4		.2	1–10	.05	12	12			6
.2	1–20	11		1	10		.05	5			5		.2	1–20	.05	11	9	2		
.2	1–40	12		1	11		.05	5			5		.2	1–40	.05	12	9	2	1	
.2	1–80	6			6		.05	5			5		.2	1–80	.05	6	3	2	1	
							.075	6	2		4									
							.1	12	12			3								

[1] + = Definite hemorrhage. [2] ± = Slight hemorrhage. [3] − = No hemorrhage.

it would indicate that the supplementary action occurred in a tumor which was resistant to filtrate. It was found that 0.05 mg. colchicine plus 0.1 cc. of filtrate produced definite hemorrhage in tumor F and complete regression in some instances. This combination, however, had no influence upon spontaneous mammary tumors.

The susceptibility of tumor F and resistance of spontaneous tumors to simultaneous injections of colchicine and filtrate led to further studies on the susceptibility of tumor F to the hemorrhage-inducing ability of filtrate. A review of earlier work up to the seventeenth passage, as indicated in table 2, revealed that strain A or ABC mice bearing 11-day-old cutaneous growths of tumor F had been injected with filtrate on seven occasions and at no time did hemorrhage occur in the tumor.

TABLE 2.—*Response of cutaneously transplanted carcinoma F to bacterial filtrate*

Serial passage number	Amount of filtrate injected	Tumor hemorrhage
	Cubic centimeters	
7	0.2, .5	Negative.
10	.5	Do.
13	.25, 2.0	Do.
14	.2, .5	Do.
15	.5, 1.0, 2.0	Do.
16	1.0, 2.0	Do.
17	.5	Do.
29	.3	Positive.
30	.5	Do.
49	.25	Do.

At this time, another experiment was performed which demonstrated conclusively the resistance of tumor F to 2 cc. of filtrate. Albino-backcross mice were inoculated cutaneously with the thirteenth passage of tumor F, and 5 days later they were inoculated cutaneously with sarcoma 37 at a site adjacent to the growth of tumor F. Six days later the mice had well-developed tumors growing side by side, and each received an intraperitoneal injection of 2 cc. of filtrate. Within 24 hours sarcoma 37 had become markedly hemorrhagic while carcinoma F was free from hemorrhage. The macroscopic observations were confirmed by histological studies.

The combined action of colchicine and filtrate in producing hemorrhage in tumor F was demonstrated in the twenty-fourth, twenty-fifth, and twenty-sixth passages of the tumor, and in each experiment 0.1 cc. of filtrate alone failed to elicit hemorrhage in control tumors.

Another experiment was performed during the twenty-ninth passage in which the control mice received 0.3 cc. of filtrate and hemorrhage occurred in their tumors. Consequently, an experiment was set up with the thirtieth passage of tumor F in which each mouse was inoculated with tumor F and sarcoma 37 and received 0.5 cc. of filtrate intraperitoneally. Hemorrhage occurred in both tumors, and the macroscopic response was confirmed by microscopic studies.

These observations indicate that between the seventeenth and thirtieth passages tumor F became susceptible to the hemorrhage-producing activity of 0.5 cc. of filtrate.

DISCUSSION

The observations recorded here confirm the findings of Boyland and Boyland (*2*) that colchicine produces hemorrhage in transplantable tumors and are in accord with those of Brues, Marble, and Jackson (*4*) that the amount capable of inducing hemorrhage in transplanted tumors is close to the lethal dose. The repeated injections of smaller doses, which failed to cause hemorrhage in propagable tumors, also failed to influence materially the growth rate of the tumors.

Colchicine and filtrate supplemented one another in producing hemorrhage and regression of transplantable mouse tumors, but their combined action was no more

effective than a single injection of colchicine or filtrate.

The combined action of colchicine and filtrate does not necessarily imply that they produce hemorrhage by acting in the same manner. Boyland and Boyland (*2*) found that the injection of colchicine or filtrate caused a reduction of the ascorbic acid content of tumors, and subsequent investigations (*9*) in this laboratory showed that parenteral administration of ascorbic acid prevented the appearance of filtrate induced hemorrhage in transplantable tumors of mice. Further studies,[1] with results similar to those of Boyland and Boyland (*10*), revealed that ascorbic acid failed to inhibit hemorrhage caused by colchicine. Colchicine, however, elicits hemorrhage only in amounts which are toxic and close to the lethal dose, while filtrate is effective in a wide range of dilutions and far below the toxic dose. It is possible that ascorbic acid inhibited the action of filtrate but not of colchicine because the action of the acid was masked by toxic doses of colchicine but was demonstrable against nontoxic amounts of filtrate.

Brues, Marble, and Jackson (*4*) suggest that large doses of colchicine may cause metabolic changes in tumors. It is possible that the combined effects of colchicine and filtrate also produce metabolic changes which result in hemorrhage. This postulation is suggested by observations recorded here which show that injection of the two substances produce toxic effects similar to a single injection of a large dose of colchicine.

The reason tumor F, upon serial passage, became susceptible to the hemorrhage-inducing activity of filtrate remains obscure. The change could not be associated with any macroscopic or microscopic alteration in the structure of the tumor or with any change in the activity of the filtrate.

[1] *Andervont, H. B., and Shimkin, M. B.: Unpublished experiments.*

SUMMARY

Colchicine when injected into mice in amounts close to the lethal dose produced hemorrhage in transplantable tumors but was no more effective than bacterial filtrate. Repeated injections of small doses of colchicine which did not cause hemorrhage in transplantable tumors failed to influence the growth of transplantable or spontaneous tumors in mice, while repeated injections of hemorrhage-inducing doses were toxic and killed the majority of animals.

Colchicine and bacterial filtrate supplemented each other in eliciting hemorrhage in grafted tumors, but their combined action was no more effective than colchicine or filtrate alone in the treatment of transplanted or spontaneous tumors in mice.

A transplantable carcinoma which in its earlier passages was resistant to the hemorrhage-inducing activity of bacterial filtrate became susceptible after the seventeenth successive passage.

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Chemical Studies on the Components of Normal and Neoplastic Tissues

III. The Composition and Amphoteric Properties of the Nucleoprotein Fraction of the Jensen Rat Sarcoma

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INTRODUCTION

The essential purpose of this series of studies consists in the characterization and subsequent comparison of certain components of tumor and normal control tissues. Studies performed on comparable normal and tumor tissues may yield some clue to the nature of the alterations from the normal to the malignant state. A number of tumors for which certain normal tissues may serve as adequate controls are at present being developed in this Institute. Until this material becomes available in quantity, we considered it of general interest to characterize the components of a few tumors for which no comparable normal tissue is known. Thus in the present communication we have studied the transplantable Jensen rat sarcoma; in the following paper (Studies IV (*1*)), we have investigated the malignant melanoma of the mouse.

The particular components of tissues with which we have been concerned have been the nucleic acids (*2*) and the proteins, with particular reference to the nucleoproteins (*3*). The present study deals with certain aspects of the nucleoprotein of the Jensen sarcoma, namely, the content of certain amino acids and the buffering or amphoteric capacities. In the latter connection, a method is described for the electrometric titration of proteins which are only partly water soluble. This method provides a new tool for the attack on the problem of the comparison of nucleoproteins from normal and from tumor tissues.

EXPERIMENTAL PROCEDURE

The tumor tissue of rats killed by decapitation was carefully dissected from the nearby muscle and from the necrotic areas. The preparation of the nucleoprotein from the tumor then followed in exactly the same fashion as the preparation of nucleoprotein from normal liver tissue described in an earlier paper in this series (*3*). All the precautions emphasized previously were followed in the preparation of the Jensen nucleoprotein. The latter material, on drying by lyophilization in the Flosdorf-Mudd apparatus formed a light nonhygroscopic, tan-colored powder. It contained considerable lipoid material, as in the case of the liver nucleoprotein (*3*). The fat was removed by treatment of the protein with a hot mixture of alcohol-acetone followed by washing with anhydrous ether. All the analytical data were obtained on the fat-free preparations.

When the protein is freshly precipitated, it dissolves at pH 8 and above, yielding a milky solution. When guanidine HCl is added to such a solution, the latter becomes clear and transparent. Thus, in its solubility behavior, the Jensen nucleo-

protein is similar to the liver nucleoprotein previously described (*3*). Solutions of the native nucleoprotein in dilute KCl–$NaHCO_3$ at pH 8, and of the nucleoprotein denatured in such solutions by the addition of concentrated guanidine HCl were used for the determination of —SH groups.

Investigations of the amino acid content of the dried, fat-free preparations were conducted according to the methods described earlier (*3*). The results are given in table 1.

TABLE 1.—*Analytical data on the Jensen tumor nucleoprotein*

[The protein was dried by lyophilization and subsequently extracted with hot alcohol-acetone-ether]

Preparation No.	N	Amide N	P	S	—SH in native protein as cysteine	—SH in denatured protein as cysteine	Cystine-cysteine	Methionine	Tyrosine	Tryptophane
	Pct.	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*
1	15.8	0.9	0.8	1.32	0.32	0.65	1.9	3.4	3.8	1.6
2	16.0	.9	.9	1.18	.23	.61	1.8	3.2	3.7	1.5

It is interesting to compare the analytical data in table 1 with the results on the liver nucleoprotein (*3*). These data on both types of nucleoprotein are comparable in magnitude for all the elements and amino acids investigated. The values for sulfur and the sulfur-containing amino acids are somewhat higher in the case of the Jensen tumor nucleoprotein, but the order of magnitude is the same. As a further striking similarity between the two types of nucleoprotein, the ratio of tyrosine to tryptophane in both is 3 molecules to 1, respectively. It would be interesting to speculate on a general similarity of structure in the animal nucleoproteins, but we have at the present time too few data to warrant further discussion of this point.[1]

[1] *Bailey (4) has shown that the myosins of a wide variety of species are very similar in composition.*

MINIMAL MOLECULAR WEIGHT OF THE JENSEN TUMOR NUGLEOPROTEIN

From the data given in table 1, the minimal molecular weight of the protein can be calculated on the basis of at least one molecule of the component with the lowest percentage existing in one molecule of the protein. Calculations on this basis are given in table 2, and apparently the minimal weight of this protein is approximately 40,000.

TABLE 2.—*The minimal molecular weight of the Jensen tumor nucleoprotein*

Component	Molecules of component	Minimal molecular weight
Sulfur	16	40,000
SH as cysteine in native protein	1	40,000
SH as cysteine in denatured protein	2	40,000
Cystine-cysteine as cysteine	6	39,996
Methionine	9	40,905
Tyrosine	9	42,840
Tryptophane	3	41,100
Average		40,691

SULFUR DISTRIBUTION AND THE DENATURATION OF THE PROTEIN

From the data on sulfur and the sulfur-containing amino acids, methionine and cystine-cysteine, it is clear that the sum of the sulfur contained in the latter two compounds is very close to the total sulfur found in the protein. The sum of methionine-S plus cystine-cysteine-S amounts to about 94 percent of the total sulfur. This indicates the absence of appreciable amounts of any sulfur-containing substances other than the amino acids mentioned.

The transition of a protein from the native to a denatured state involves an intramolecular rearrangement of unknown nature. This phenomenon is often accompanied by the appearance of certain reactive chemical groups in the denatured protein, and the number of these newly formed groups may serve as an indication

of the extent of the denaturation process (*5*, *6*, *7*). The most readily detectable of these groups is the sulfhydryl group which belongs to the amino acid cysteine. The first attempts to measure quantitatively the amount of mercaptan groups which appeared in proteins as a consequence of denaturation were made by Mirsky and Anson (*8*) and by Todrick and Walker (*9*). These workers used various oxidants as specific titrating agents in heterogeneous mixtures of heat-coagulated proteins. Greenstein showed later that the amount of mercaptan groups which appeared as a result of denaturation was dependent upon the method of denaturation employed, and further that the most powerful denaturing agents for all the proteins investigated, i. e., those which liberated the most —SH groups, were the guanidine halides (*5*, *6*, *7*, *10*). These observations were afterwards confirmed by Anson (*11*), whose results on egg albumin were similar within experimental error to those of Greenstein.[2]

The general method for estimating the presence of the mercaptan groups in proteins has involved titration with specific oxidizing agents. Anson and Mirsky (*8*) used cystine, phosphotungstic acid, iodine, and ferricyanide. Todrick and Walker (*9*) used dichlorophenolindophenol. Kuhn and Desnuelle (*13*) used the dye porphyrindin which Greenstein (*5*, *6*, *7*, *10*) later adapted in conjunction with nitroprusside. All of these reagents can be standardized by the use of a cysteine solution of known concentration. Most of the recent work on a wide variety of proteins has been performed with the porphyrindin- nitroprusside technique described in considerable detail by Greenstein and Edsall (*5*, *6*, *7*, *10*). As a result of these and other studies, it appears that proteins may tentatively be divided into four separate categories. These classes are: (1) Proteins with negative tests for —SH groups in the native state but positive tests in the denatured state; (2) proteins negative for —SH in both native and denatured states; (3) proteins which in the native state are positive for —SH and which show an increase in —SH on denaturation; and (4) proteins which have the same proportion of —SH groups in both native and denatured states.

Proteins in class 1 include egg albumin (*5*, *6*, *7*, *8*), edestin (*5*, *6*, *7*), excelsin (*5*, *6*, *7*), globin (*5*, *6*, *7*), papain (*14*), and serum albumin (*5*, *6*, *7*). Proteins in class 2 include amandin (*5*, *6*, *7*) and insulin (*5*, *6*, *7*). Proteins in class 3 include myosin (*10*). Proteins in class 4 include the liver nucleoproteins (*3*). It is evident that proteins either in the native or in the denatured form may vary widely in their sulfhydryl composition.

From the data in tables 1 and 2, it is apparent that the nucleoprotein of the Jensen tumor belongs to class 3. The proportion of cysteine in the native protein is 0.23–0.32 percent. On treatment of a solution of the native protein with 10 M guanidine HCl, thus denaturing the protein, this value about doubles. The cysteine was estimated in the solutions by the

[2] *The use of porphyrindin has been criticized by Anson (11), who in a brief note unsupported by any experimental evidence stated that the dye is "hard to make, unstable, and a dangerously powerful oxidant." It might be stated that the synthesis of porphyrindin is not particularly difficult, nor is the dye at all unstable if it is pure. That its oxidizing powers may be misused, is illustrated by the procedure of Brand and Kassel (12). These investigators attempted to titrate the —SH groups of egg albumin by adding a huge excess of the dye and estimating the amount of dye unconsumed. Far more dye than corresponded to the maximum number of mercaptan groups in the protein was consumed. This experiment (12) indicated that under conditions involving the presence of a large excess of the dye, groups in the protein other than mercaptan will be oxidized. Such conditions are clearly inapplicable to these studies. The essence of any specific chemical method of the type used in these studies is the avoidance of undesirable side reactions. The stepwise procedure for the titration of —SH groups with porphyrindin until the point is just reached where the nitroprusside reaction disappears, described in detail by Greenstein and Edsall (10), appears to be reliable and capable of yielding reproducible results.*

porphyrindin-nitroprusside method previously fully described (*10*).

The cysteine revealed in the denatured protein amounts to one-third of the total cystine-cysteine content of the protein (tables 1 and 2). If all the cysteine were revealed by the denaturation, it would indicate that of the six atoms of cystine-cysteine sulfur in the protein of minimal weight 40,000 (table 2), two of them belonged to two molecules of cysteine and four belonged to two molecules of cystine. By the use of the denaturation procedure it is thus possible to obtain some idea of the relative distribution of cystine and cysteine in the protein.

THE AMPHOTERIC PROPERTIES OF THE JENSEN TUMOR NUCLEOPROTEIN

The acid and alkali which may be bound to the protein molecule at varying pH values are most readily determined by the method of electrometric titration. A considerable literature has developed on this subject, and has been critically reviewed by Cohn (*15, 16*) and by Cannan (*17*).

Briefly, the method of electrometric titration involves the portion-wise addition of standard acid or alkali to a solution of the protein, followed by the determination of the pH of the solution after each addition. The pH may be measured by any number of means, the most customary being by the hydrogen or the glass electrode. The acid or alkali which is bound to the protein is equal to the difference between the measured amount of acid or alkali added to the protein and the amount of free hydrogen or hydroxyl ions as measured by the pH. The acid or alkali is a concentration factor; pH, however, is an activity factor, and in order that it be expressed in terms of concentration, the activity coefficient must be known. These considerations may be illustrated by the following expressions: the acid or alkali bound, B, may be defined in solution containing acid or alkali, or both, by

$$B=[K^+]-[Cl^-]+[H^+]-[OH^-] \qquad (1)$$

The last two terms are calculated from the following relations:

$$pH=-\log (H^+) \qquad (2)$$
$$(H^+)=[H^+] \times \gamma H^+ \qquad (3)$$
$$Kw=[OH^-] \times \gamma OH \times (H^+) \qquad (4)$$

The activity coefficients, γ, in equations 3 and 4 are either taken from the literature or are calculated from pH determinations of appropriate solutions of HCl or of KOH. We have used the convention in equations 1–4 of enclosing activity factors in parentheses and concentration factors in brackets. The calculations of B are made on the assumption that $[Cl^-]$ and $[K^+]$ are equal to the concentrations of added acid and of added alkali, respectively. This, of course, implies that the only ion which combines with, or dissociates from the protein is H^+ or OH^-.

A further, very important assumption is that the value of γ, the activity coefficient in equations 3 and 4, is the same in water and in the protein solutions (*15*). The activity coefficient for an ion in any event is largely an arbitrary quantity because of the interposition of a liquid junction potential of unknown magnitude. This has been discussed by a number of workers (Cannan (*17*)) and need not be further discussed here. For the purposes of the present investigation, we have assumed the validity of the equations 1–4, while recognizing the difficulties involved in such an acceptance.

The plot of pH against B provides the titration curve of a protein, yielding the same information as the comparable curves of weak acids and of amino acids and peptides (*18*). Thus at the points of inflection along such curves, we may cal-

culate empirically the values of dissociation constants characteristic of certain chemical groups which dissociate within a particular range of pH (Cohn (*15*)). The extreme ends of such curves, which should approach a limiting value of *B* in acid and alkali, yield information concerning the maximum amount of acid and of alkali which is bound by the protein(*15*). In such plots, *B* is generally expressed as the acid or alkali bound per milligram of protein. The values of the acid and of the alkali bound are an expression of the total amphoteric character of the protein. The dissociation constants at the points of inflection along the curve reveal the specific buffering capacity of the protein at particular pH ranges.

THE TITRATION CURVE OF JENSEN TUMOR NUCLEOPROTEIN

In the attempt to conduct a complete electrometric titration study of this or other nucleoproteins, a peculiar difficulty arises. Nucleoproteins in general are soluble in water at pH values of 8 and above. Below pH 8 they become progressively insoluble, so that within this range there is a mixture of soluble and insoluble protein until a pH of about 3 to 4 is reached where all of the protein is insoluble. While it is equally possible to titrate either a completely soluble protein or a completely insoluble protein over the entire range in pH, as Steinhardt and Harris (*19*) have shown, the titration of a protein whose solubility changes with every change in pH presents problems at present difficult to overcome. A titration curve can, of course, be constructed under such conditions (*20*), but it has very little meaning.

The problem, therefore, resolves itself into the finding of a suitable solvent for the nucleoproteins so that they may be titrated over the entire pH range. We have found that certain proteins, including the type under consideration, may be kept in solutions of guanidine HCl under conditions which would render them insoluble were they in water alone. It is true, of course, that these proteins were denatured (*3, 10*). We have at present no information as to how denaturation will affect the amphoteric properties of any protein. While the use of solutions of the nucleoproteins in guanidine HCl might provide a method for titrating them in continuous fashion over the entire pH range, we thought it desirable to investigate first the effect of the guanidine HCl on the titration of a protein which is completely soluble in water. The titration curves of such a protein in water and in guanidine HCl when compared should reveal the effect of the denaturant. For this purpose we chose beef serum albumin which was purified several times. The concentration of guanidine HCl was 6 M.

The use of guanidine HCl imposes a certain amount of caution. This is a neutral salt, but the guanidine ion in solutions of strong alkali decomposes into ammonia. In order to avoid such an occurrence, and also to avoid the possibility of irreversible changes taking place in the protein in alkaline solution (*19*), we conducted the titrations throughout at 0° C. The guanidine HCl was purified by dissolving in a large amount of dry methanol, filtering, and then crystallizing the salt from the filtrate by the addition of anhydrous ether.

The electrometric assembly included a Leeds and Northrup type K potentiometer with box galvanometer. The hydrogen electrode was of the point type and was freshly platinized and saturated with hydrogen before every run. Care was taken to design the electrode to avoid any dead spaces. The electrode was inserted into a cell of about 50-cc. capacity.

The cell was closed by a four-holed rubber stopper through which the electrode, the outlet tube, the burette, and the agar bridge were inserted. Thus at no time during the titration were the contents of the cell exposed to air. The agar bridge was connected with a beaker of saturated KCl into which the calomel electrode dipped. The entire electrode assembly was mounted in a bath of cracked ice and water which kept the temperature at 0° ± 0.01° C.

The calomel electrode was standardized against 0.10 N HCl, and a pH for the latter of 1.08 (*19*) was assumed. For the estimation of the activity coefficient of OH^- in water, a pH of 13.56 for 0.05 N KOH was assumed (*19*). The activity coefficients of H^+ and OH^- in 6 M guanidine HCl were estimated from the pH values of 0.1 N HCl and 0.05 N KOH, respectively, in the presence of this salt; pKw at 0° C. was taken as 14.94.

The burette, which read to 0.005 cm., was filled with either 1.0 N HCl or 1.0 N KOH and inserted into the stopper of the titrating vessel. Twenty cubic centimeters of a 2.60-percent solution of the beef serum albumin was placed in the cell. For the titrations in the presence of guanidine HCl, 11.46 gm. of the salt was added to the 20 cc. of solution and the mixture gently shaken to solution. To prevent frothing a drop of caprylic alcohol was added. The entire apparatus was then assembled, and the potentiometer readings were taken after the addition of measured quantities of acid and of alkali. It was found that whereas equilibrium was rather slowly reached in alkaline solutions in water, it was very rapidly attained in alkaline solutions in guanidine HCl. On the acid side, equilibrium at the electrode was rapidly attained in either water or in guanidine HCl. The reaching of equilibrium in alkaline solutions in guanidine HCl is an indication that there is no decomposition of the salt.

The results of the titration are given in table 3, and the titration curve is graphically illustrated in figure 1. The abscissa in figure 1 is the pH value; the ordinate is the acid or base bound per milligram of protein, calculated from *B* in equation 1.

Inspection of figure 1 reveals three interesting points of comparison between the titration of the protein in water and in 6 M guanidine HCl: (1) The total acid plus base combining capacity of the protein is

TABLE 3.—*Titration of beef serum albumin in water and in 6M guanidine HCl*

[20.0 cc. of solution containing 540 mg. of protein was used. The denaturant, 11.46 gm., was added to this solution. Titrations were conducted at 0° C. Calculations were made on the basis of 20.0 cc. H_2O at start of titration]

In water		In 6M guanidine HCl	
Acid or alkali bound per milligram protein	pH	Acid or alkali bound per milligram protein	pH
$mM \times 10^3$		$mM \times 10^3$	
84.4	1.92	93.7	1.51
84.3	2.07	93.3	1.63
83.5	2.26	93.9	1.86
80.4	2.51	92.8	2.02
76.8	2.72	90.4	2.52
69.2	2.90	82.4	3.08
63.3	3.05	75.4	3.41
56.1	3.20	65.2	3.69
49.4	3.35	54.8	3.94
41.8	3.52	43.3	4.17
34.1	3.74	34.4	4.39
30.7	3.80	24.6	4.64
27.0	3.90	18.3	4.84
23.7	4.01	10.0	5.19
20.0	4.12	3.5	5.63
16.5	4.26	0	6.02
12.8	4.46	8.1	6.83
10.9	4.53	11.8	7.18
9.1	4.66	17.4	7.89
5.4	4.96	20.9	8.75
3.5	5.12	24.6	9.29
1.6	5.31	28.1	9.58
0	5.52	32.6	9.79
4.4	6.08	35.4	9.95
9.1	6.81	41.1	10.23
20.9	8.48	46.3	10.43
24.6	9.18	51.3	10.58
27.2	9.53	56.8	10.74
33.5	9.76	61.5	10.88
40.7	10.68	65.9	11.03
48.5	11.05	72.0	11.22
58.3	11.56	77.6	11.39
69.8	11.83	81.7	11.58
80.6	12.08	85.0	11.71
89.1	12.19	87.0	11.84
97.6	12.41	87.0	12.03
98.1	12.78	86.5	12.15
97.0	13.04	89.1	12.36
		89.5	12.51

the same in water and in guanidine HCl, but the alkali binding is greater in water and the acid binding is greater in guanidine HCl; (2) the dissociation constants estimated from the points of inflection on the curve are three in number in water and two in number in guanidine HCl; and (3) the values of the dissociation constants are whereby they are transformed from alkali-binding groups in water to acid-binding groups in guanidine HCl. Thus, there is an increase of 7.5×10^{-3} mM in acid binding and a corresponding decrease of 7.5×10^{-3} mM in alkali binding, on denaturation in guanidine HCl. This equivalence in loss and gain is to be expected if the total number of groups remains the same, but it is impossible at the present time to do more than speculate about the cause of the shift. The "loss" of these groups for base-binding purposes in guanidine HCl is reflected in the fact that there is a decided change in the shape of the titration curve in the denaturant on the alkaline side; thus, whereas there are two points of inflection with dissociation constants of pK 9.5 and pK 11.7 in water there is only one such constant, pK 10.6,

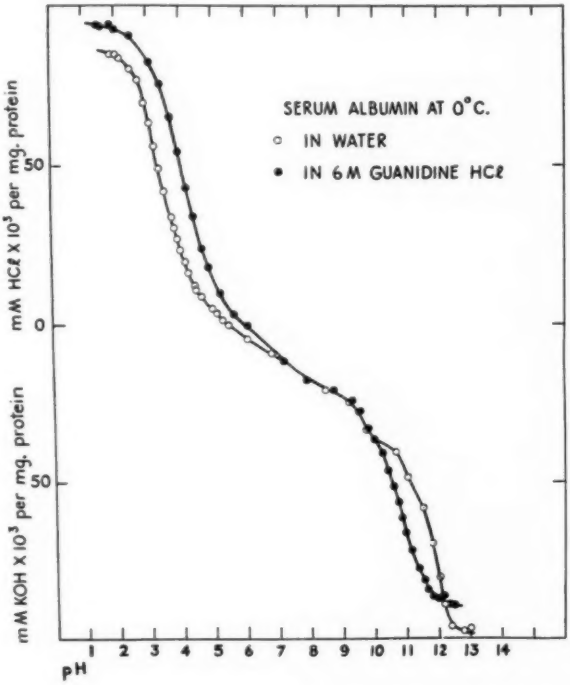
SERUM ALBUMIN AT 0°C.
IN WATER
IN 6M GUANIDINE HCl
mM HCl X 10³ per mg. protein
mM KOH X 10³ per mg. protein
50
0
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14
pH

FIGURE 1.—The titration curve of beef serum albumin in water and in 6M guanidine HCl at 0° C.

○—in water; ●—in 6M guanidine HCl.

quite different for the protein in water and in guanidine HCl.

The fact that the total acid-and-base-binding capacity is the same in water and in the denaturant is not altogether surprising. Presumably the total number of dissociating chemical groups in the protein is not altered as a result of denaturation. However, as reference to table 4 shows, there is an apparent change in the character of certain groups, equivalent in amount to 7.5×10^{-3} mM per milligram of protein,

in guanidine HCl. While the curves on the acid side may both be described in terms of a single dissociation constant, the pK of this constant in water is 3.7, in guanidine HCl it is 4.4.

TABLE 4.—*Acid- and alkali-binding capacities of serum albumin and of the Jensen tumor nucleoprotein*

Protein	Solvent	HCl bound per milligram protein	KOH bound per milligram protein	Total
		$mM \times 10^3$	$mM \times 10^3$	$mM \times 10^3$
Serum albumin	H_2O	88.0	100.0	188.0
Do	6M guanidine HCl	95.5	92.5	188.0
Jensen tumor nucleoprotein	6M guanidine HCl	119.5	150.0	269.5

It is evident, therefore, that there is a notable effect of guanidine HCl on the protein whereby the structure of the protein is altered. From the work discussed earlier in this paper and recent studies on this protein (*5*, *6*, *7*), it appears that —SH groups are revealed in serum albumin as a result of treatment with guanidine HCl. These groups cannot have been formed *de novo* as a result of the denaturation, or else the total number of groups after denaturation would be greater than that before denaturation. Moreover, such groups, if formed *de novo*, would cause an increase in the alkali-binding capacity of the protein in guanidine HCl, rather than a decrease, such as actually occurs. The appearance of these —SH groups on denaturation is only a single aspect of the intramolecular change which accompanies this process.

The problem of interpretation of these results is complicated by the fact that the guanidine HCl plays a dual role in its effect on the protein, for it acts both as a neutral salt and as a denaturant. The effect of a neutral salt on the titration curve of a protein is to narrow the range of dissociation over that in pure water, i. e., the acid branch of the curve becomes more alkaline, and the alkaline side becomes more acid. This phenomenon is clearly shown by the experiments of Cannan (*17*) and of Steinhardt and Harris (*19*), and is demonstrated in the present study in figure 1. On the other hand, the "loss" of a point of inflection and the changes in the relative amounts of acid- and alkali-binding capacities are not encountered in the neutral salt effect and must, therefore, be ascribed to the denaturing effect of the guanidine salt.

The total acid-and-base-binding capacity of serum albumin appears to be unaltered as a result of denaturation in guanidine HCl. For the time being we shall assume that this fact holds for other proteins and shall employ this titration method in guanidine HCl as one more tool in the investigation of the nucleoproteins from various tissues. The present results on the nucleoprotein of the Jensen rat sarcoma are reported in table 5 and are graphically presented in figure 2.

The method of using this protein in these

TABLE 5.—*Titration of Jensen tumor nucleoprotein in 6M guanidine HCl*

[20.6 cc. of solution containing 393 mg. of protein and 8.186 gm. guanidine HCl was used. Titrations were conducted at 0° C. Calculations were made on the basis of 14.2 cc. H_2O at start of titration]

Acid or alkali bound per milligram of protein	pH	Acid or alkali bound per milligram of protein	pH
$mM \times 10^3$		$mM \times 10^3$	
116.0	0.50	12.2	4.51
116.2	.62	30.0	5.06
115.5	.75	38.7	5.51
114.5	.99	49.1	6.56
106.6	1.07	61.1	7.76
102.9	1.12	71.2	9.20
100.0	1.18	82.9	9.81
98.9	1.27	92.6	10.23
94.4	1.34	103.6	10.54
92.4	1.46	112.5	10.76
86.3	1.58	120.9	11.02
83.7	1.81	135.2	11.56
74.8	2.03	140.7	11.88
67.1	2.66	140.7	12.06
52.1	3.29	139.0	12.20
35.1	3.66	144.0	12.45
17.8	3.94	143.0	12.62
0	4.23		

studies consisted in washing the freshly prepared and freshly precipitated protein with ice-cold distilled water many times at the centrifuge until salt-free. A suspension of the protein in water to the total volume of 50 cc. was then made and 28.65 gm. of guanidine HCl added. A drop of caprylic alcohol was added and the mixture shaken gently to solution. The total volume of the solution was 70.0 cc. With the exception of a very slight residue, the protein was completely dissolved. The residue was centrifuged off, and 20 cc. of the clear supernatant used for the titration with 1.0 N HCl, and another 20 cc. with 1.0 N KOH. The concentration of protein in these aliquots was determined by diluting 10 cc. of the stock solution to 200 cc. with distilled water. The protein precipitated completely thereby, was centrifuged, washed with water, alcohol, and ether, and was finally dried and weighed. The protein concentration was then estimated on the basis of the original suspension in pure water, i. e., as if it had not been a suspension but a true solution in water. Calculations of combining capacity were thus based on 14.2 cc. of water at the start of the titrations.

The acid- and base-binding capacities of this protein were calculated from figure 2 and are given in table 4. It is apparent that the total capacity, namely, 269.5 $\times 10^{-3}$ mM per milligram protein is considerably larger than the corresponding value of 188.0 $\times$ 10^{-3} for the serum albumin. This would indicate the presence of far more dissociable chemical groups in the nucleoprotein as compared with the serum protein. The former protein has a high amphoteric capacity. Points of inflection on the curve in figure 2 reveal dissociation constants of pK 1.3, 1.9, 4.2, and 10.6. The latter two constants are strikingly similar to the two pK values of 4.4 and 10.6 found in the serum albumin de-

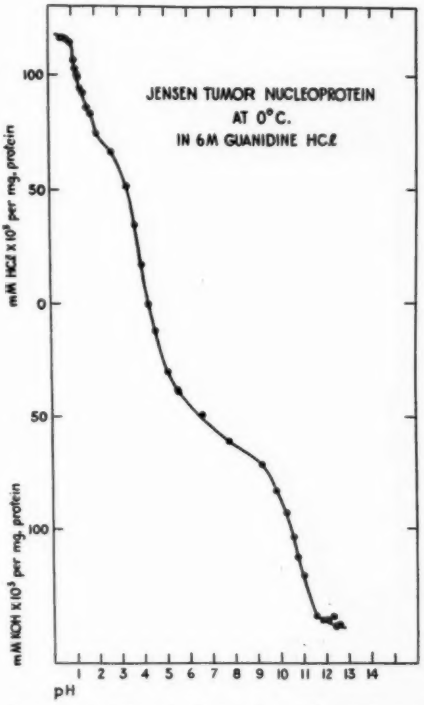

FIGURE 2.—The titration curve of the Jensen tumor nucleoprotein in 6M guanidine HCl at 0° C.

natured likewise in 6M guanidine HCl. Further discussion, however, of the phenomena elicited as a result of denaturation will be postponed until more titration data on various proteins in guanidine HCl become available. The total acid-and-base-binding capacity can, however, be used directly as a basis of comparison between various proteins under the same conditions.

SUMMARY

1. The nucleoprotein of the Jensen rat sarcoma has been prepared by the method previously described for the preparation of the liver nucleoprotein.

2. Analytical data on the nucleoproteins are reported. The proportion of amino

acids is similar to that previously reported for the liver nucleoprotein. Calculation of the minimal molecular weight of the Jensen tumor nucleoprotein from the analytical data reveals a value of about 40,000.

3. The sulfur distribution and the behavior of —SH groups on denaturation are discussed for this protein. The latter contains free —SH groups in the native state, and the number of these groups is increased on denaturation by guanidine HCl. From these data the relative distribution of cysteine and of cystine may be inferred.

4. The amphoteric properties of the nucleoprotein have been investigated by potentiometric titration studies in 6M guanidine HCl. The results are evaluated on the basis of comparable studies on serum albumin in water and in guanidine HCl. From these studies it appears that the total acid-and-base-binding capacity is the same in the native and denatured states of the protein but that denaturation imposes certain profound alterations in the protein structure which are reflected in differences in the pH range of dissociation of chemical groups in the protein. The Jensen tumor nucleoprotein possesses a very considerable capacity to combine with acid and with alkali, indicating the presence within the protein of a number of reactive chemical groupings.

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Chemical Studies on the Components of Normal and Neoplastic Tissues

IV. The Melanin-Containing Pseudoglobulin of the Malignant Melanoma of Mice

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INTRODUCTION

From the time of Berzelius, the chemistry of the melanotic pigment has been of interest both to the biochemist and to the cancer specialist, and a considerable literature has been built on this subject (*1*). Indeed, tumor tissue, chiefly in the form of metastatic growths in the liver, has served the biochemist as one of the most readily available natural sources of this pigment. Other sources have included the choroid and retina of the eye. The interest in this pigment arises from its possible relationship to the metabolic transformations of tyrosine and 3,4-dihydroxy phenylalanine (*1*). These amino acids in the pure state give rise by enzymic oxidation to a black pigment similar in certain respects to the melanotic pigment isolated from normal and tumor tissues. The extensive studies of Raper and his associates (*2*) have been concerned with the pigment arising from the pure acids. Studies of the pigment from various tissues have been made by a number of workers; their analytical data have been summarized by Waelsch (*3*). The results for the same tissue melanin have varied considerably, the nitrogen values ranging from 8.4 to 13.7 percent, the sulfur values ranging from 0 to 11.3 percent. Such variations are quite unsatisfactory and are probably caused by incomplete or too drastic methods of separation of the pigment from the tissue. Thus many workers have used pepsin-HCl which yields an incomplete digestion of the tissue proteins; or else they have used hot alkali which might conceivably result in decomposition of labile sulfur linkages. A milder method for the isolation of the pigment is obviously demanded if the analytical values are to be acceptable as a basis of comparison between the melanins from different sources. However, any attempt to isolate the pigment from the tissue directly gives no clue to its mode of combination within the tissues. Here particularly information is very desirable.

Since presumably the pigment is involved in certain phases, normal or pathological, of protein metabolism, its relation to the proteins of the tissues in which it is found is worthy of investigation. There are no data available on the proteins of melanotic tissue. We therefore began the present investigation by fractionating the proteins of the malignant melanoma of the mouse. It was found that approximately 99 percent of the proteins of this tumor consist of a water-soluble pseudoglobulin which furthermore appears to have attached to it practically all of the tumor melanin. The properties of this protein were investigated from the stand-

points of composition and viscosity behavior. The melanin was separated from the protein by a method involving an enzymic degradation of the protein. A batch of melanin was isolated after 1 month's digestion of the protein with pancreatin at pH 7.8. Investigation of the composition of the protein fragment still attached to the melanin gave some clue to the points of attachment of the pigment to the protein.

EXPERIMENTAL PROCEDURE

MELANOMA S–91

The tumor used was originally found by Dr. Arthur M. Cloudman of the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. It grew spontaneously at the base of the tail of a female strain dba mouse as a firm, black, hairless growth. After histopathological confirmation, he called it melanoma S–91 and made the first transplant on July 12, 1937. When received at the National Cancer Institute on November 15, 1939, it was in the twelfth generation transplant, in the strain of mice in which it spontaneously developed.

In this Institute the tumor has been further successfully transplanted subcutaneously in strain dba mice. Both tumor-fragments and tumor-mash techniques were used. No regressions of well-developed transplanted tumors have been observed either by Dr. Cloudman or by us.

THE FRACTIONATION OF THE PROTEINS OF THE MELANOMA

The fresh tumor material after careful dissection from 20 or more mice was pooled and frozen overnight. The tissues were then ground by machine and extracted for 4 hours with gentle stirring with 3 times the amount of a chilled solution of 0.5 M KCl and 0.03 N $NaHCO_3$. The pH of the mixture was 7.8. At the end of the stirring, the mixture was filtered either through paper pulp or fine gauze, and the filtrate treated with powdered ammonium sulfate to 0.8 saturation. The dark-colored protein precipitated in bulk within a few minutes and was filtered off. The filtrate was colorless and completely free of protein. The protein was scraped off the filter and transferred to a cellophane dializing bag, and dialyzed against cold water in the ice chest for several days until SO_4-free. The bag was then opened and the black-colored mixture centrifuged. Surprisingly, there was only a minute amount of insoluble material. Treatment of this residue with dilute KCl solution caused the solution of little more than a trace of euglobulin. The greater part of this small residue appeared to be either denatured protein or tissue debris. By far the greater amount of the protein from the melanoma tissue was water-soluble.

In order to determine what proportion of this water-soluble protein was albumin or pseudoglobulin, powdered ammonium sulfate was added to 0.4 saturation. Practically all of the protein was thereby precipitated. Addition of ammonium sulfate to the filtrate from this precipitate resulted in a faint, whitish haze, but no observable precipitation. It would appear, therefore, that the protein composition of this melanoma tissue in curious fashion is distributed in a trace of euglobulin and albumin, while the greater bulk consists of pseudoglobulin. Three separate preparations were made from various lots of this tumor, and each time the same distribution was observed. In the case of two of these preparations, the aqueous solution of the pseudoglobulin was treated with an excess of pure dioxane and the precipitate allowed to stand. The latter was then washed several times with dioxane, then several times with ether, and finally dried at room temperature in vacuo for several days. The euglobulin

of one of the preparations was extracted with KCl from the residue following the dialysis (see above), and was dried with alcohol and ether. The dried pseudoglobulin formed a grayish-black powder, the euglobulin was grayish-white. It seems probable, from the color and relative proportion of the protein fractions, that the melanin pigment is entirely or nearly entirely associated with the pseudoglobulin fraction.

The yield of the dried melanin-containing pseudoglobulin was 2.5 to 4 percent of the total wet weight of the tissue. The trace of albumin described above was not measurable. The proportion of euglobulin, estimated with some difficulty because of its small amount, appeared to be about 0.05 percent of the total wet weight of tissue. Thus the pseudoglobulin fraction forms well over 99 percent of the total proteins of this tumor.

THE AMINO ACID COMPOSITION OF THE PSEUDOGLOBULIN

The further investigation of the melanoma proteins was confined to the melanin-containing pseudoglobulin. The first studies were concerned with the proportion of several of the amino acids in the protein. Most of the methods of analysis have been described in an earlier paper in this series (*4*) and will not be repeated here. Cystine-cysteine was determined by the Folin-Marenzi method. Because of the color of the melanin, methionine could not be determined in the protein by means of the method involving the opening of the thiolactone ring, and hence the volathe iodide method was used. Results are given in table 1.

There is a relatively high proportion of sulfur in this protein. The sum of cystine-S plus methionine-S amounts to about 70 to 73 percent of the total sulfur. Whether this indicates the presence of sulfur-containing substances other than cystine and methionine, or whether the presence of melanin in the hydrolytic digests of this protein interferes with the quantitative determination of the amino acids cannot be decided at the present time. In any event, sulfate-S appears to be absent. Phosphorus is also absent.

TABLE 1.—*Composition of the melanin-containing pseudoglobulin of the mouse melanoma*

[The protein was dried with dioxane and ether and kept in an evacuated desiccator for several days over phosphorus pentoxide]

Preparation No.	N	P	Amide N	S	SO_4S	Cystine-cysteine	Methionine	Tyrosine	Tryptophane
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1	15.0	0	1.0	1.6	0	2.6	2.0	2.7	1.0
2	14.7	0	.9	1.5	0	2.4	2.1	2.8	1.0

MINIMAL MOLECULAR WEIGHT OF THE PSEUDOGLOBULIN

From the analytical data given in table 1, the minimal molecular weight of the protein may be estimated. The results are collected in table 2, and it may be seen that this protein apparently possesses a minimum molecular weight of about 20,000. The actual molecular weight will be some multiple of this, and plans for an ultracentrifugal analysis of this protein are already under way.

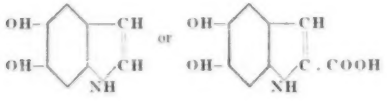
TABLE 2.—*Minimal molecular weight of the pseudoglobulin*

Amino acid	Mols	Calculated minimal molecular weight
Tyrosine	3	19,740
Tryptophane	1	19,608
Cystine-cysteine	[1] 4	19,608
Methionine	3	20,700
Sulfur	10	20,000
Average		19,931

[1] As cysteine.

THE MELANIN ASSOCIATED WITH THE PSEUDOGLOBULIN

It appears likely that practically all of the melanin in the tumor is associated with the pseudoglobulin fraction. In what manner is the pigment attached to the protein? Is it in the nature of a prosthetic group, bearing a possible relation to the pseudoglobulin as heme does to globin, or is the pigment actually an integral part of the protein molecule, bound in polypeptide linkage? If tissue melanin is in any way similar to the products obtained by the action of tyrosinase on tyrosine (*2*), namely:



it would be difficult to see how such substances could be bound within the polypeptide chain by the usual type of linkage. Or else it is possible that tissue melanin may be formed by alterations of tyrosine bound within the polypeptide chain. The latter explanation, by analogy, appears to be unlikely; whereas many specific enzymes are known which act upon a number of the free amino acids, none of them affect the acids when the latter are bound in the protein. On the other hand, both tissue melanin and the melanin derived from the action of tyrosinase on tyrosine appear to be colloidal polymers of undetermined molecular weight. Such polymers might readily combine with proteins either by virtue of intermolecular attractive forces or else by combination through one or more specific groups. The combination of polysaccharides with proteins is an illustration. In such conjugated proteins, the prosthetic group may be either loosely or relatively firmly bound to the protein, the degree of binding often depending upon the nature of the protein.

The only work in determining the nature and composition of the protein residue has been done by Waelsch (*3*). This investigator isolated melanin from melanotic metastases to the liver and from cattle eyes by prolonged pepsin-HCl digestion of the ground tissue.

If one tentatively assumes that the melanin pigment in tissues is analogous in structure to that formed by the action of tyrosinase on tyrosine, it would be expected that the former pigment would approach the latter, not only in colloidal properties but also in elementary composition. The pigment formed from tyrosine has a nitrogen value of 8.6 percent (*2*). The pigment isolated from tissues has been reported by several investigators to vary from 8.4 to 13.6 percent in nitrogen. Moreover, the pigment from tyrosine contains no sulfur, whereas the tissue melanin reported by nearly all investigators contains sulfur in amounts ranging as high as 11.0 percent. It is obvious that in all these preparations a residue of protein has still remained attached to the melanin. The characterization of this residue is of considerable importance for it indicates the approximate location on the protein where the pigment is attached, i. e., the protein "environment" of the pigment.

We preferred to isolate the melanin from the pure protein, which provides the possibility of a cleaner separation from a substance of ascertained composition than from the ground tissue such as Waelsch used. Furthermore, pancreatin was used as the enzyme for it carries protein digestion further than pepsin does, and the hydrolysis could be conducted at a nearly neutral reaction.

Four grams of the dried pseudoglobulin were suspended in a solution of 0.5M KCl and 0.03N $NaHCO_3$ (pH 7.8) and treated with 0.5 gm. of pancreatin and a few drops of toluol. The mixture was

set in an incubator at 40° C. for 1 month, the toluol being renewed two or three times. At the end of this time the mixture was filtered through paper pulp and then dialyzed against distilled water for 1 week in the ice chest. No diffusion of the pigment occurred. The contents of the dialysis bag were centrifuged from a minute amount of suspended matter. The black-colored solution was then treated with 10 percent acetic acid to pH 5. The melanin precipitated completely. It was washed at the centrifuge several times with very dilute acetic acid. The use of slightly acidulated water is necessary, for the washing of the melanin with water alone will cause the formation of a colloidal suspension. Finally the pigment was dried with alcohol and ether. The yield was 390 mg., or 9.7 percent of the protein used. The melanin was a jet black material like coal dust. It was analyzed in the same fashion as the protein from which it was derived. The data are collected in table 3.

TABLE 3.—*Melanin separated by pancreatin digestion from the pseudoglobulin*

N	S	Cystine-cysteine	Methionine	Tyrosine	Tryptophane
Percent 11.0	*Percent* 2.5	*Percent* 1.4	*Percent* 8.9	*Percent* 1.9	*Percent* 1.3

For the cystine-cysteine analysis, 50 mg. of the pigment were used, and 18.4 mg. were recovered following the usual hydrolysis with 20 percent HCl at 110° C. for 16 hours. This recovered material gave 8.8 percent nitrogen on analysis, and no sulfur.

It is clear that starting with a protein containing 1.5–1.6 percent sulfur, a considerable enrichment in sulfur-containing compounds attached to the melanin is achieved by the enzymic degradation of the protein. Whereas the protein itself contains about 2 percent of methionine, the pigment obtained has nearly 9 percent of this amino acid. The sum of the cystine-cysteine-S and the methionine-S is nearly equal to the total S of 2.5 percent found in the pigment. The ratio of tyrosine to tryptophane in the melanin has changed from that in the protein with the result of a greater enrichment in tryptophane as compared with tyrosine. It is, however, the magnitude and the distribution of sulfur in the pigment which is rather remarkable, and with some justification it might be assumed that at the point or points of attachment of the pigment to the protein there is a relatively high proportion of the sulfur-containing amino acids. We still do not know how the pigment is attached to the protein but we now have some idea of what its neighbors are. Other amino acids than those analyzed for are unquestionably present, i. e., a positive Sakaguchi reaction for arginine was given, but further investigation will be postponed until more material is available.

The melanin isolated by enzymic digestion of the pigment-containing pseudoglobulin may provisionally be designated as a polypeptide to which about one-half of its weight of pigment is attached. When the polypeptide is completely hydrolyzed by HCl, the pigment remaining has a nitrogen value of 8.8 percent which is characteristic of that found in the pigment formed from tyrosine.

THE VISCOSITY OF THE PSEUDOGLOBULIN

Further characterization of the chief protein of the tumor was considered desirable. Since viscosity measurements provide some indication of the shape of protein molecules (*5*), such studies were carried out with the pseudoglobulin. The methods employed for this investigation have been described in some detail in earlier papers of this series (*4*, *6*). The

viscosity of the protein solution in water and in urea and guanidine salts was measured under various velocity gradients. In order to obtain a solution of protein of sufficiently high concentration, the pseudoglobulin after the final dialysis (see above) and centrifuging was concentrated under reduced pressure in a new and simple type of ultrafilter, which is described in the section following. The viscosity data are given in table 4 and are illustrated in figure 1. The symbols used have been described in detail before (*6*).

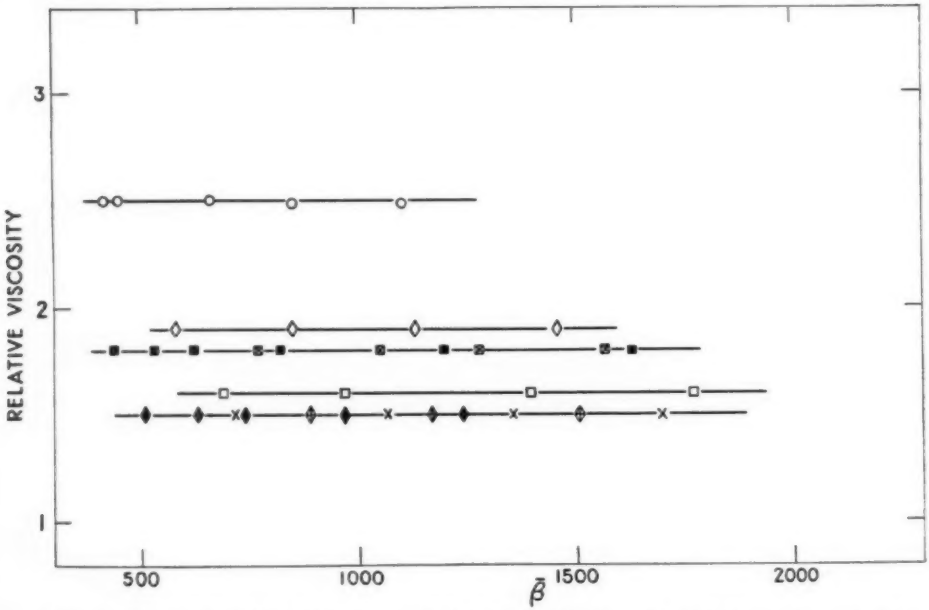


FIGURE 1.—The viscosity of the melanoma pseudoglobulin as a function of the velocity gradient. O—in water; ◇, ⊕, ◆—in 1M, 4M, and 8M urea, respectively; □, ⊠, ■—in 1M, 4M, and 8M guanidine HCl, respectively; X—in 1M Na I.

It is interesting to note that the viscosity of the protein is independent of the velocity gradient, and one would be inclined on this basis to classify the pseudoglobulin among the globular proteins for which this relation generally holds. The viscosity of the fibrous proteins, such as myosin, is quite dependent on the velocity gradient (*5*). A further differentiation of these two classes of proteins lies in the observation that denaturing agents such as urea and guanidine HCl generally cause an increase in the viscosity of the globular proteins and a decrease in the viscosity of the fibrous proteins (*5*, *6*, *7*). Thus, if the pseudoglobulin here considered were really a globular type of protein it might be expected that the addition of urea and guanidine HCl would cause an increase in its viscosity. Actually, however, the addition of these substances causes a considerable decrease in its viscosity (table 4 and fig. 1). Moreover, the addition of these salts beyond 4M in concentration has no further effect on the viscosity, whereas in the case of all the proteins (and nucleic acid) investigated in solutions of these

TABLE 4.—*Viscosity of the pseudoglobulin in water and in salts at 30° C.*

[The concentration of protein was 2.75 percent]

Salt	Concentration of salt in mM added per cubic centimeter solution	Specific viscosity	ν_r
H_2O	.....	1.50	77
Urea	1.0	.91	47
Guanidine HCl	1.0	.61	31
NaI	1.0	.53	27
Urea	4.0	.53	27
Guanidine HCl	4.0	.81	42
Urea	8.0	.52	27
Guanidine HCl	8.0	.82	42

salts, the maximum effect on the viscosity had apparently not been reached even at 8M (*4*, *5*, *6*).

On the other hand, it is interesting to note that guanidine HCl at 1M concentration lowers the viscosity of the protein to a greater extent than at 4M and 8M concentrations. It seems likely that the shape and size of the protein moleules are different in 1M guanidine than they are in 4M or 8M guanidine. These findings, together with the data in urea solutions, may be further tentatively interpreted in the following way: The pseudoglobulin in water is globular in shape. When guanidin HCl is added to 1M concentration, the protein is dissociated into smaller, more symmetrical particles. Such a change would account for the drop in viscosity in this solution. The addition of more guanidine HCl to 4M concentration causes an alteration in the apparent shape of these particles in the direction of greater asymmetry. This results in an increase in the viscosity (*8*, *9*). Addition of more guanidine HCl (to 8M) produces no further effect. Urea, on the other hand, is apparently capable only of causing dissociation of the pseudoglobulin into smaller, more symmetrical particles and exerts no further effect on these particles. Urea at 4M concentration decreases the viscosity of the protein by about two-thirds, an effect which is accomplished by sodium iodide at 1M concentration. The order of effectiveness of these denaturing salts is the same as that found in the case of other proteins (*4*, *5*), and of nucleic acid (*6*).

There is independent evidence to support the concept of the dissociation of globular proteins by denaturing agents. Burk (*10*) has shown by osmotic pressure experiments that edestin, excelsin, and amandin are dissociated into smaller particles by urea. Steinhardt (*11*) has shown by ultracentrifugal measurements that hemoglobin is split into molecules of half the original molecular weight by the action of urea and other amides. Measurements of the colligative properties of proteins under these conditions reveal the size of the molecules; viscosity measurements are indicative of shape. We have some idea of the possible alterations in the shape and size of the melanoma pseudoglobulin under various conditions of denaturation, but a clearer and more definite picture can be obtained only when the present results are correlated with data derived from experiments conducted under these conditions in the ultracentrifuge. Plans for a study of this sort are already under way.

A SIMPLE ULTRAFILTER

Most of the ultrafilters in use with protein solutions employ collodion for the membrane. This substance is quite useful when the pore size is to be regulated. However, for ordinary ultrafiltration of most proteins, cellophane provides the simplest and the most readily prepared material. Its elasticity is not as great as that of collodion and care must be taken to provide a jacket little larger than the tube of cellophane to be employed. We have found the following type of ultrafilter to be very simple in assembling and satisfactory in performance. The framework consists of a glass tube of

1-inch inside diameter, sealed at one end and fitted with a sidearm 3 inches from the open end. The sidearm is connected through a trap to an oil pump. The closed ends of several Soxhlet extraction thimbles of 1-inch diameter are cut off and the paper cylinders inserted one on top of the other into the glass tube, reaching from the bottom of the tube to well beyond the sidearm. Two or three glass rods, carefully polished on the ends, are inserted between the paper walls and the glass in such a way that they cover the aperture leading to the side tube. The rods thus prevent the drawing of the paper wall into the side tube when suction is applied. Then a wetted piece of $36/32$ Visking cellophane tubing is tied off at one end and inserted within the paper-lined glass tube. Into the open end of the cellophane tube a No. 5 double-holed rubber stopper is inserted and the open end of the glass tube sealed with the cellophane-covered stopper. A small glass funnel is set into one of the two holes of the stopper. The protein solution is then poured through the funnel into the dialysis bag and the suction at the pump started.

An example of the use of the apparatus is afforded by the data on a solution of the melanoma pseudoglobulin. The solution contained 1.37 gm. of protein in 100 cc. volume. After 4 hours of ultrafiltration at 25° C. and 12 mm. Hg pressure, 50 cc. of water, completely free of protein, had passed through the membrane.

SUMMARY

The proteins of the malignant melanoma of mice were fractionated. It was found that well over 99 percent of the proteins of this tumor consisted of a pseudoglobulin fraction. A trace of albumin and little more than a trace of euglobulin were present.

The pseudoglobulin contained practically all of the melanin found in the tumor. The conjugated protein was analyzed for the elements and for certain amino acids. Calculation based on these data indicated a minimum molecular weight of this protein of about 20,000.

The melanin was removed from the protein by pancreatin digestion at pH 7.8 for 1 month, and was analyzed in a similar fashion to the parent protein. The results indicated that protein material was still bound to the pigment, this material being particularly rich in sulfur-containing amino acids. The process of digestion resulted in an enrichment of sulfur in the protein fragment attached to the pigment, and one might conclude that at the point or points of attachment of the pigment to the pseudoglobulin a great number of sulfur-containing amino acids are concentrated.

The viscosity behavior of the pseudoglobulin in water and in denaturing salts indicates that the protein is of the globular type and is dissociated by the salts into smaller, more symmetrical fragments.

A simple type of ultrafilter is described.

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The Influence of Genetic Constitution Upon the Induction of Resistance to Transplantable Mouse Tumors[1]

By Morris K. Barrett, *research fellow, National Cancer Institute, National Institute of Health, United States Public Health Service*

The induction of resistance to transplantable tumors in mice by a prior inoculation of homologous normal living cells was an early contribution to the study of cancer. Bashford, Murray, and Cramer (*1, 2, 3, 4*) reported the use of defibrinated blood to induce resistance by this method. Although it has long been recognized that the injection of homologous living cells has no effect on the development of spontaneous (*5, p. 84*) or induced (*6, p. 18*) tumors, nor will it influence the course of an established transplantable tumor (*7*), repeated attempts have been made to relate this phenomenon to control of the natural disease.

The mechanism involved has been the subject of extensive investigations, but no generally accepted explanation has been evolved. Murphy (*7*) has suggested that the phenomenon may be due to a type of sensitization and has adduced some evidence in support of such a conception. According to this view, the initial injection of normal cells sensitizes the animal to the subsequent inoculation of tumor cells in such a way that the resisting mechanisms of the host, whatever they may be, are caused to act with enhanced force. Whether this represents a cellular immunity in the ordinary sense remains an open question, but it should be kept in mind that the "sensitization" here is directed against certain attributes of intact cells which depend upon their viability and integrity, and is not just a matter of specific proteins or other cell constituents. This interpretation presupposes a degree of genetic difference between the cells injected and the host as a basis for the reaction. Such opinion is supported by the failure to stimulate resistance by injection of autologous cells (*8; 9, p. 150*) or to influence the take of an autograft by prior injection of homologous normal cells (*10; 11, p. 540*). Furthermore, the injection of defibrinated blood of an alien species will not influence the transplantation of mouse tumors into mice (*12*).

Now that strains of mice are available which are relatively, if not entirely, homogeneous, it is possible to test the effect of genetic differences upon this phenomenon and to make some inferences with regard to the influence of genetic homogeneity. It is important to do so because there is little chance of applying the principles involved to the control of the naturally occurring disease, if the mechanism proves to be a sensitization phenomenon.

If leukemia represents essentially the same pathologic process as cancer, a view now taken by many, there already exist some studies along these lines. Rhoads and Miller (*13*) showed that resistance to transplantable leukemia may be induced in mice by the injection of intact homologous cells; and MacDowell, Potter, and Taylor (*14*) presented evidence that, when embryonal tissues were used as the

[1] *This work was carried on in the laboratories of Dr. James B. Murphy of the Rockefeller Institute for Medical Research.*

immunizing agent, the degree of resistance induced is dependent upon the genetic relationship of the donor and the recipient. These latter workers adduced further that the effect attained was not directly related to the susceptibility of the strain from which the donor came.

The work presented here was undertaken in order to observe some effects of genetic differences upon the phenomenon under discussion. This was done by comparing the growth of grafts of three transplantable mammary carcinomas in mice of three strains. Test mice of each strain were immunized with blood of their own strain, or with that of the other strains, and the results compared with each other and with thos obtained with control animals. The material selected is not ideal for complete elucidation of the problem, but the results are significant and dependable within their range. The technique used was simple and is described in the following section.

MATERIALS AND METHODS

Young animals of the Rockefeller Institute strain, of Strong's A strain, and of the C57 Black strain were selected as hosts. The Rockefeller Institute strain has been derived by mass inbreeding of an original stock comprising approximately 600 animals equally distributed among 3 albino strains—the Lathrop, the Bagg, and the Uppercu. All were obtained in 1917, and no new animals have been added since. The Rockefeller Institute strain was taken as a genetically indifferent strain which presented, nevertheless, much uniformity. The strain A mice were obtained in weekly consignments from the Roscoe B. Jackson Memorial Laboratory. The strain is a highly inbred albino one that has undergone brother-by-sister inbreeding since 1918. The C57 Black mice were also obtained from the Roscoe B. Jackson Memorial Laboratory. This is a strain which has been inbred by brother-sister matings for more than 20 generations. However, it is possible that animals from more than 1 subline were included in those used.

All hosts were from 4 to 6 weeks old, and their average weight was 12 to 16 gm. Most of the animals were males, but the sexes were not segregated.

Three transplantable mammary carcinomas were employed. These were (1) the well-known Bashford No. 63, here taken as a genetically indifferent tumor; (2) the tumor known as 15091–A, a low-factor tumor which arose in an A strain female at Bar Harbor and is transplantable outside the parent strain (Cloudman (*15*)); and (3) tumor 755 which originated in a C57 Black mouse in the colony of Dr. H. J. Bagg, to whom the writer is indebted for this material. The tumor last mentioned was ill-suited to this work but was used in the absence of better material. It could not be used outside the strain of origin but did give some information within that strain.

Blood was obtained, with aseptic precautions, from the heart of old adults of each strain and defibrinated by shaking with glass beads. The nuchal region of the host was shaved, and 0.2 cc. of the defibrinated blood was injected subcutaneously.

The mice of each strain were divided into groups by random selection. The first group of each was given blood taken from A mice, the second group received blood from Rockefeller Institute mice, the third group received blood from C57 Black mice, and the fourth or control group received no blood. Some groupings were omitted in individual experiments for lack of material. Precautions against subconscious bias in observing the results were taken by indexing the random distribution on a separate record which was not again consulted until the completion of the experiment.

After the passage of 10 days all mice received a graft (approximately 3×3×2 mm.) of healthy carcinoma tissue inoculated by means of a trocar into the groin. The growth of the grafts was recorded weekly in terms of caliper measurements. Final results were evaluated after 2 weeks' growth. The tumors were observed for 2 weeks beyond the initial period in all cases, but the figures given below reflect the number of tumors present at 2 weeks. Little or no difference would be seen if calculations were based upon a longer period of observation; but since the investigation here is limited to the initial establishment of a graft, it was thought that the earlier data served the purpose best. This is in keeping with a belief that, though there may be common factors, the primary take of a transplantable tumor is not necessarily dependent upon the same factors as is the subsequent growth of the tumor.

The work includes 27 experiments, using a total of 768 animals as hosts.

RESULTS

The results obtained revealed wide fluctuations in susceptibility of the animals. The variations are such that, even in this somewhat limited material, almost the entire range of susceptibility is covered.

Table 1 summarizes the data on all mice that were grafted with tumor 15091–A, the A strain tumor. In Rockefeller Institute mice grafted with this tumor the variations were very marked. It will be noticed that the greatest resistance was found in those animals which had been immunized with blood homologous to the tumor. For convenience the word "homologous" is used throughout the remainder of this paper in the special sense of "having the same or similar genetic constitution."

Under the heading "Strain A" in table 1 are shown the results of inoculation of immunized animals of an inbred strain, A, with a tumor (No. 15091–A) which originated in that strain. The variation shown is not significant statistically. It may be observed that when genetically homogeneous mice are used, resistance to a homologous tumor cannot be induced by this method.

TABLE 1.—*Results of inoculating immunized and control mice with tumor No. 15091–A (an A strain tumor)*

Strain used as source of blood for immunization	Total mice	Susceptible		Resistant	
	No.	*No.*	*Pct.*	*No.*	*Pct.*
ROCKEFELLER INSTITUTE MICE					
A	30	2	7	28	93
Rockefeller Institute	30	5	17	25	83
C57 Black	29	22	76	7	24
None (Controls)	29	29	100	0	0
STRAIN A MICE					
A	30	28	93	2	7
Rockefeller Institute	29	28	97	1	3
C57 Black	30	30	100	0	0
None (Controls)	30	30	100	0	0
C57 BLACK MICE					
A	28	4	14	24	86
Rockefeller Institute	30	2	7	28	93
C57 Black	29	25	86	4	14
None (Controls)	28	27	96	1	4

The results of inoculating C57 Black mice with tumor 15091–A are also given in table 1. This represents the case of a pure-line host with a tumor which originated in a different pure line. The resistance induced by blood which is homologous to the tumor, i. e., strain A blood, is again great though not quite so great as that induced by Rockefeller Institute blood. However, the difference between these two is not significant statistically ($P=0.34$), and the important observation is the effect of the A blood. It is interesting to note here that, according to Strong, one of the ancestral parents of the A strain was a Bagg albino, a strain which also enters into the Rockefeller

Institute strain. It is also noted that in these relatively pure line hosts the homologous blood had little or no effect in inducing resistance against a heterologous tumor.

Table 2 presents the results of transplanting a genetically indifferent tumor (Bashford No. 63) into an indifferent strain (Rockefeller Institute) and into a homogeneous strain (A).

TABLE 2.—*Results of inoculating immunized and control mice with Bashford No. 63 (a genetically indifferent tumor)*

ROCKEFELLER INSTITUTE MICE

Strain used as source of blood for immunization	Total mice	Susceptible		Resistant	
	No.	*No.*	*Pct.*	*No.*	*Pct.*
A	44	2	5	42	95
Rockefeller Institute	48	14	29	34	71
None (Controls)	49	24	49	25	51

STRAIN A MICE

A	83	50	60	33	40
Rockefeller Institute	88	34	39	54	61
None (Controls)	74	58	78	16	22

It will be seen that in strain A mice the percentage of takes in the group given blood from the same strain was reduced to 77 percent of that in the controls while the incidence in the group immunized with blood from the other strain was reduced to 50 percent of that in the controls. In the Rockefeller mice the incidence of takes in the group immunized with blood from the same strain was reduced to 59 percent of that in the controls, whereas a comparable figure for the group given blood from the other strain was 10 percent. It should be noted that the effect of homologous blood, as measured by relative reduction in percentage of takes, was less in the case of the A strain.

The results of inoculating C57 Black mice with tumor No. 755 are given in table 3. Here again is evidence that no effective resistance can be induced in a pure-line mouse against a tumor that is genetically homologous to the host.

TABLE 3.—*Results of inoculating immunized and control C57 Black mice with tumor No. 755 (a C57 Black tumor)*

Strain used as source of blood for immunization	Total mice	Susceptible		Resistant	
	No.	*No.*	*Pct.*	*No.*	*Pct.*
A	10	9	90	1	10
C57 Black	10	10	100	0	0
None (Controls)	10	10	100	0	0

Careful analysis of the data failed to disclose anything in the size of the tumors which could be correlated with the treatment of the animals, nor was anything of interest discovered by noting the rate or the time of death among the animals in groups having a high incidence of takes.

Statistical analysis of the data indicates that the differences observed were definitely significant.

DISCUSSION

It must be emphasized that the discussion here is limited to influence upon the establishment of a graft. It is believed that after this initial period, when the tumor has become established, a new set of conditions prevail and that the factors governing the two periods may differ in part, if not altogether. This is not to say that there is no relation between the phenomenon under discussion and certain aspects of the natural disease, e. g. extension and metastasis.

All the data given in the tables are summarized in figure 1. All results in a given strain of mice are arranged in a horizontal row, while similar categories within strains are arranged in vertical columns. Some interesting differences can be seen at a glance.

It is apparent that, with the methods employed here, the genetic interrelationships of the materials used have an important influence upon the degree of resistance

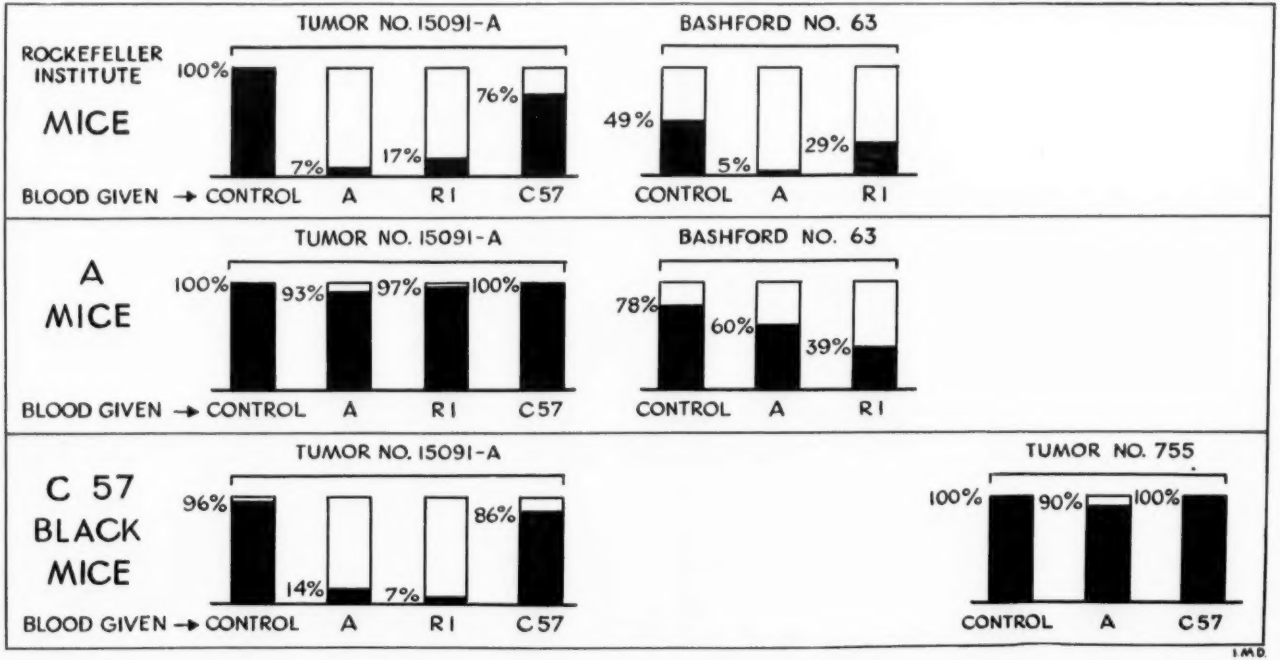

FIGURE 1.—Summary of results in all mice. Individual columns represent all mice in a category. Shaded portions represent susceptible animals, and clear portions resistant animals. Percentages are shown at the left of each column. The groupings show difference of host or tumor. All mice in the first row are of Rockefeller Institute strain; in the second row, A strain; and in the third row, C57 Black strain.

induced. The variations are so great that, even in this limited material, one may select conditions which will give almost any degree of resistance, ranging from nearly complete immunity to complete susceptibility. The inferences from this with regard to other types of experiments in which these factors may be operative are obvious and somewhat disturbing. Herein lies a part of the importance of this work; the factors which are demonstrated here may affect certain types of experiments which have no primary relationship to this field, for these influences may be operative in any experiment which involves the transfer of living cells from one host to another.

An inspection of figure 1 reveals that by using the same mouse and the same tumor, high resistance or almost no resistance may be obtained according to the blood used. Or, if the tumor and the immunizing blood are kept constant, the response varies with the host. Again, if the host and blood are kept constant, the response varies with two different tumors. The degree of response elicited by a given blood does not seem to be related to the susceptibility of the strain from which the blood was derived, but the data available do not provide sufficient evidence for a decision as to possible intrinsic qualities of the blood. From these results, however, one might say, upon limited evidence, that when the blood is closely related to the tumor but not to the host, a high degree of resistance will be attained.

Considering the results obtained with the Bashford tumor, there is a reduced incidence of takes in both strains given either blood, but for either strain the reduction is greater when the alternate blood is used as the immunizing agent than when the corresponding blood is given. This relation is also true in the group of C57 Black mice inoculated with tumor 15091–A. It does not hold in the case of Rockefeller Institute mice inoculated with tumor 15091–A, where C57 Black blood conferred least resistance. For lack of a better explanation this last will have to be ascribed to variability in the animal stocks and treated as an exception until more evidence is obtained.

These relations favor the belief that the mechanism involved is a sensitization type of phenomenon, in the sense suggested above, and depends upon the foreignness of the tissues used. If one accepts this viewpoint, the results are confirmatory since it seems obvious that the cells (i. e., the blood cells) of the alternate strain are more foreign to any host than are the cells of the corresponding strain, and the cells of the tumors in each of these cases are foreign to the host. Further support is given to this viewpoint by the failure to induce resistance in either the A strain or the C57 Black strain against a tumor of homologous origin. This result is but a step removed from Haaland's reported failure to induce resistance against an autograft.

Such a line of reasoning leads to the thought that, as the foreignness of the immunizing dose toward the host is decreased, there should follow a corresponding decrease in the degree of resistance induced. The final step in this series is, of course, the injection of autologous cells, which is known to be without effect. The data here may present an intermediate step since the relative reduction in the percentage of takes produced by giving a mouse blood from an individual of its own strain is less in the case of the more inbred, and supposedly more homogeneous, strains (i. e., Little's A and C57 Black).

Thus the evidence obtained by these experiments together with that of previous workers gives force to the most important implication of this work, which is—if the

considerations above are correct—that the search for a means of treating human cancer based upon these principles (e. g., autoplastic grafting, sensitizing injections, etc.) is likely to prove a fruitless one.

Many unknown factors are involved in these relationships, and the final solution of the problem waits upon more data. Further work along these lines is being undertaken.

SUMMARY

It has been shown that when resistance to transplantable tumors is induced in susceptible mice by subcutaneous injection of defibrinated whole blood, the degree of resistance varies with the genetic interrelationships of the host, the tumor, and the donor (of the blood).

With the two pure-line tumors used, no significant resistance could be induced in an inbred mouse against a tumor derived from the same strain.

The implications of these findings are discussed.

Acknowledgment is due Dr. James B. Murphy, in whose laboratory this work was done, for helpful cooperation.

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Convenient Inexpensive Device for Quantitative Hand Feeding of Mice

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In controlled experimental feeding of mice, it is often essential to get the mouse to ingest definite quantities of drugs, dietary supplements, or other materials. The mouse does not readily accept food from a pipette or syringe [1] as does the rat, nor does it ingest satisfactorily supplements fed separately. Furthermore, it is not always desirable, or possible, to mix these supplements with the other dietary constituents.

A very simple device for the purpose of feeding mice definite amounts of material has found wide application in the National Cancer Institute. It consists of a 1-cc. tuberculin syringe which is fitted with a modified 18-gauge curved hypodermic needle with a slightly enlarged tip to prevent injury to the soft tissue of the mouth and throat. The needle is made as follows: A drop of silver solder (ordinary solder is too soft) is applied circumferentially just back of the bevel of a 2-inch 18-gauge needle. It has been found that a strip of this solder $\frac{1}{16}$ of an inch wide, cut from a $\frac{1}{4}$-ounce sheet of silver solder and sufficiently long to wrap twice tightly around the needle, is most satisfactory. After the solder is wrapped around the needle, it is then treated with a drop of flux made of zinc chloride in dilute hydrochloric acid and dried above the flame of a microburner. After drying, the part to be soldered is placed at the peak of the reducing portion of the flame of the microburner and held there for several seconds until the silver is securely welded to the needle. After cooling, this solder is smoothed with a file until it makes only a slight enlargement near the end of the needle (fig. 1). The tip of the needle is then cut off flush with the outer edge of the solder, and smoothed and rounded off with file and fine sandpaper. After the tip has been prepared, the needle is bent to the correct angle as shown in figure 1 by heating to a dull red heat in a microburner and quickly bending to the correct shape with a pair of pliers.

The successful use of this method of feeding depends on holding the mouse firmly, yet in such a way that the animal will not struggle. Experience has shown that this is best accomplished by having the mouse on a surface where it can secure a toehold, then grasp it by the base of the tail with the little finger pressed against the palm of the hand and by the skin in the region of the neck just posterior to the ears, with the index finger and the thumb as shown in figure 2. The mouse is held with head up as illustrated, the needle is passed over the tongue, at the same time the animal's head is pushed upward and slightly backward, and the needle is passed through the pharynx and into the esophagus. The required dosage is then given. If the end of the needle lies in the esophagus a distance of approximately 2 cm. from the tooth margin in an adult mouse, no regurgitation results, and it is possible for one person with an assistant to feed mice almost as rapidly as the animals can be caught. No injury has been observed from many feedings in this way.

Mice have been fed daily vitamin supple-

[1] *Nelson, Dorothy: The administration of drugs to rats. Science, 92: 92 (1940).*

FIGURE 1.—Tuberculin syringe, fitted with hypodermic needle, used in hand-feeding experiments with mice.

ments for 6 months or more in this laboratory with no ill effects from the use of the feeding device. Very small mice can be fed in this way, too, if the operator is careful. When the mice weigh from 5 to 8 gm., it is best to use a 20-gauge needle and keep the angle of the curved portion approximately the same as that of the larger needle.

FIGURE 2.—Proper way to hold mouse to get best results in hand-feeding experiments with mice.

Sunlight and Cancer of the Skin

By HAROLD F. BLUM, *research fellow, National Cancer Institute, National Institute of Health, United States Public Health Service*

The idea that sunlight is an important causal agent in cancer of the skin is not new. In a treatise on histopathology of skin diseases, written nearly 50 years ago, Unna (*1*) describes "seaman's skin" as a precancerous condition attributable to continued exposure to light, and ascribes to the same agent an inciting role in xeroderma pigmentosum, an inherited malignant disease of the skin. Other early exponents were Dubreuilh (*2*, *3*), Shield (*4*), Hyde (*5*), and Bellini (*6*). At present, few writers oppose the view that sunlight may be an etiological factor in cutaneous cancer, but opinions vary with regard to its relative importance. This is exemplified in two recent monographs on tumors of the skin by Mackee and Cipollaro (*7*) and Eller (*8*); the one frequently indicts sunlight as a causal factor, while the other refers only occasionally to this agent.

Until quite recently the evidence has been entirely clinical, that is, it has consisted of attempts to correlate clinical findings with extent of exposure and susceptibility to sunlight. The following conclusions have been reached by various workers: (1) Cancer of the skin occurs principally on parts exposed to sunlight; (2) cancer of the skin is more prevalent in outdoor workers; (3) the incidence of cancer of the skin is greater in regions of the earth which receive greatest insolation; and (4) cancer of the skin occurs more often in blonds than in brunets. These arguments seem valid enough at first, but on critical examination become somewhat less convincing. Their weakness lies chiefly in failure properly to evaluate the sunlight factor, which may be credited in great part to paucity of information regarding the incidence of sunlight upon, and penetration into, the skin. Much necessary information is still lacking, so that even today any analysis of this phase of the subject must be open to question.

Within little over a decade strong support has come to the theory of direct causation of cancer by sunlight, from experiments on laboratory animals. During this period a number of independent workers have produced tumors of the skin by the action of ultraviolet radiation. These studies are not only important because they demonstrate that cancer can be produced by radiation of wavelengths present in sunlight, but also because they yield information which permits better evaluation of the evidence obtained from clinical study.

This review has been undertaken with the hope that the examination of the interrelations of these various lines of evidence may provide a better understanding in the prosecution of further studies.

ANIMAL EXPERIMENTS

Credit for first producing tumors in laboratory animals by mercury arc radiation belongs to Findlay (*9*), who described his experiments on mice in 1928. Within the 4 years following the announcement of his first experiments, reports of similar findings appeared from three other laboratories, where similar investigations were going on at about the same time: Putschar and Holtz (*10*) and Roffo (*11*) using rats, and Herlitz, Jundell, and Wahlgren (*12*) using mice. All four studies were conceived independently, no group of workers

being aware at the inception of its own investigations that similar experiments were going on elsewhere.

Successful experiments have also been carried out by Huldschinsky (*13*), Beard, Boggess, and Von Hamm (*14*), Rusch and Baumann (*15*), and Rusch, Kline, and Baumann (*16*). The incidence of tumors may be very high, some workers reporting that 100 percent of surviving animals developed neoplasms.

In 1934 Roffo (*17, 18*) showed that natural sunlight as well as mercury arc radiation may cause tumors in rats.

DISTRIBUTION AND TYPES OF TUMORS

As might be expected, the tumors arise principally in those regions which have least hair, and hence are more directly exposed to the radiation. The ears, nose, tail, paws, and artificially depilated areas are most commonly affected. Both carcinomas and sarcomas are reported. Papillomas are frequent in occurrence.

THE ACTIVE WAVELENGTHS

The wave lengths which cause tumor formation are restricted to the same spectral region that is active in sunburn and in antirachitic action. The observations from which the limits of the action spectrum (see p. 412) for tumor production may be deduced are listed in table 1. These come from four different laboratories. Only those of Rusch, Kline, and Baumann (*16*) include sufficient description of the spectral nature of the radiation and of the filters to permit accurate delimitation of the spectrum. However, general knowledge of the spectral characteristics of sources and filters permits the assignment of approximate wavelength limits for the observations of other workers, and when this is done essential agreement between the four sets of data is apparent.

The experiments are arranged in the table according to the source employed and are best discussed in this order. The first group of experiments comprises those in which intermediate pressure mercury arcs of the "therapeutic" type were used. Such areas emit discrete lines of a limited number of wavelengths in the ultraviolet and visible regions of the spectrum. The positions of the ultraviolet lines important to the present discussion are shown in

TABLE 1.—*Experiments determining the wavelengths active in tumor production*

Group	Investigators	Animal	Source	Filter	Approximate wavelengths [1]	Tumor production
I	Rusch et al. (*16*)	Mouse	Hg arc in quartz [2]	None	Longer than 2250A	+
		do	do [2]	Corex D	Longer than 2800A	+
		do	do [2]	Window glass	Longer than 3200A	0
	Roffo (*17, 18*)	Rat	do [2]	None	Longer than 2250A	+
		do	do [2]	"Transparent glass" [3]	Longer than 3200A	0
II	Roffo (*17, 18*)	do	Sunlight	None	Longer than 2900A	+
		do	do	"Transparent glass"	Longer than 3200A	0
III	Beard et al. (*14*)	do	SI [4]		Longer than 2800A	+
	Findlay (*19*)	do	Tungsten filament	None	Longer than 4000A	0
	Roffo (*17, 18*)	do	do	do	do	0
	Rusch et al. (*16*)	Mouse	do	do	do	0
IV	Rusch et al. (*16*)	do	Low pressure Hg arc in quartz.		2537A [5]	0
V	Roffo (*17, 18*)	Rat	"Neon lamp"		Many lines in ultraviolet and visible.	0
		do	"Radio waves"		0.3 to 20 meters	0

[1] These are in most cases estimates by the writer, based on the general characteristics of sources and filters. (See text.)
[2] Intermediate pressure or "therapeutic" type mercury arc.
[3] Also numerous colored glasses all of which probably had a window-glass base.
[4] This combines tungsten filament and mercury-arc radiation, probably in a Corex envelope.
[5] 89 percent at this wavelength.

figure 1. Intensities for an arc of this type are also indicated, as are transmissions in the short wavelength cut-off regions for filters of the types used.

From Rusch, Kline, and Baumann's experiments (*16*) in the first group, it is apparent that only mercury lines of wavelength 3130A or shorter, cause tumor production since the mercury arc radiation that passes through window glass is ineffective. Window glass of ordinary thickness removes the 3130A and all shorter lines. Roffo's (*17*, *18*) "transparent glass" was almost certainly window glass, and hence his observations are in agreement with those of Rusch, Kline, and Baumann. The latter found that mercury arc radiation passing through Corex D was about as effective as unfiltered radiation. Under their conditions, using the Corex D filter, virtually all the energy of wavelength shorter than 2967A was eliminated.

Roffo's experiments with sunlight form the second group in table 1. He found that total sunlight was effective in producing skin tumors, but that sunlight passing through "transparent" glass was not. The shortest wavelength in sunlight is about 2900A; and, assuming that Roffo's "transparent" glass was window glass, the longest wavelengths of sunlight effective must be in the neighborhood of 3200A. This agrees with the findings for the mercury arc. Furthermore, this experiment provides evidence that wavelengths longer than this limit do not cause tumor formation. The mercury arc emits relatively little energy in the longer wavelengths as compared with sunlight, and this might account, conceivably, for failure

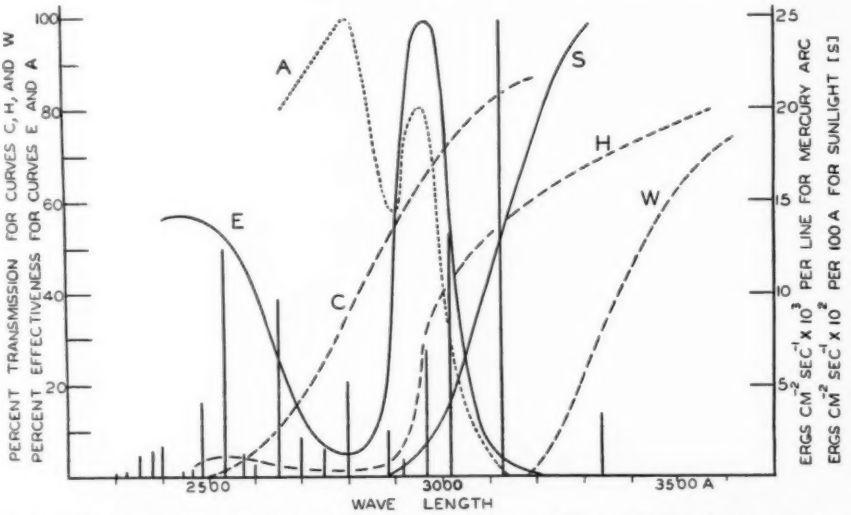

FIGURE 1.—*E*, action spectrum (the relative effectiveness of different wavelengths) for erythema of sunburn (Coblentz, Stair, and Hogue (*20*)); *A*, action spectrum for antirachitic action (Knudson and Bendford (*21*)); *S*, spectrum of sunlight reaching the earth at summer solstice, noon at Tucson, Ariz. (Pettit (22); *H*, transmission of various wavelengths by human epidermis 0.08 mm. thick (Lucas (*23*)); *C*, transmission by a Corex D filter; and *W*, transmission by window glass. The vertical lines represent the wavelengths and the energies of the lines of a mercury arc of the intermediate pressure or "therapeutic" type (McAlister(*24*)).

to produce tumors with mercury arc radiation filtered through window glass. On the other hand, reference to figure 2 will

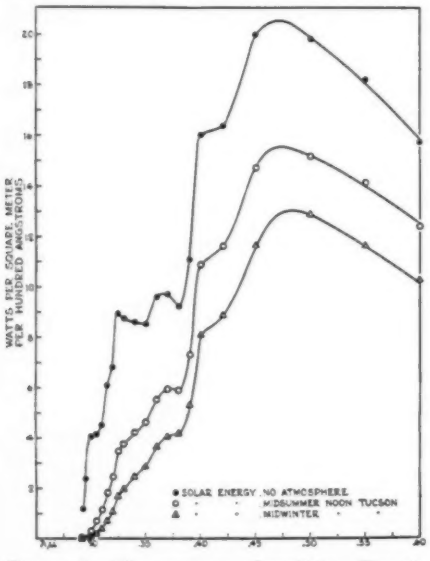

FIGURE 2.—The spectrum of sunlight. The abscissa is wave lengths in r; multiply by 10,000 to obtain A. From observations at Tucson, Ariz. (Reproduced by permission of Pettit (*25*).

show that the portion of sunlight energy of wavelengths longer than 3200A is many times that of shorter wavelengths. Some of this radiation was, of course, lost by absorption and reflection by the glass filter, but the evidence that the longer ultraviolet, visible, and infrared radiation of sunlight is ineffective in tumor formation, seems conclusively established by Roffo's experiments.

The next group of experiments (III) adds to the evidence on this point. All the sources used were tungsten-filament lamps, which emit most of their radiation in the visible and infrared. In the experiments of Beard, Boggess, and Von Hamm, a General Electric SI lamp was used, which emits mercury arc radiation in addition to the radiation from the tungsten filament, and in this case tumors were produced. See Luckiesh (*26*) for a description of this type of arc.

Rusch, Kline, and Baumann (group IV) found that a low-pressure mercury arc, which emits virtually all its radiation at wavelength 2537A did not cause tumor formation. This agrees with their finding that the radiation from an intermediate pressure mercury arc was about as effective when filtered through Corex D, as when unfiltered; and seems to set the short wavelength limit for tumor production at about 2900A. A glance at figure 1 will show that transmission of the epidermis falls off rapidly in the region of 2900A, and suggests that the short wavelength limit may be set by this factor. (See p. 413.)

Group V includes miscellaneous experiments which add little to the evidence, but on the other hand do not contradict any of the other findings.

Miescher (*27*) states that Funding, Henriques, and Reckling have reported experiments with filters which showed that the tumor-producing wavelengths are shorter than 3200A, but gives no details of the experiments. This agrees with the experiments cited in table 1, and there can be little doubt that 3200A is about the long wavelength limit for tumor production in laboratory animals.

Reference to figure 1 and the above discussion will suggest a descrepancy between the wavelengths that produce tumors, and those which cause antirachitic effects and the erythema of sunburn. This apparent discrepancy will be discussed below. (See p. 413.) For the intervening discussion it will be sufficient to recognize the fact that the tumor-producing wavelengths in sunlight are limited to a very restricted portion of the whole, namely, those wavelengths lying between about 3200A and the short wavelength limit

at 2900A. For purposes of discussion it will be assumed that the wavelengths for tumor production in laboratory rodents are those incriminated in the production of cancer in man.

ENERGIES REQUIRED FOR TUMOR PRODUCTION

In the preceding discussion no mention has been made of the actual energies required for tumor production. Information regarding this is lacking in the earlier studies, but Rusch, Kline, and Baumann have measured the intensities of radiation in their experiments, and estimate the total energy required for tumor production. This varies somewhat according to the conditions of the experiment and the strain of mice used. Their lowest value is 6.3×10^6 ergs per square centimeter per day for 42 days, or a total of 2.6×10^8 ergs per square centimeter of the tumor-producing wavelengths.[1] In this case the first tumor did not appear until 112 days after the radiation was discontinued. These values are for albino mice, and must be higher for C57 Brown mice. (See below.)

An estimate of the incident solar energy in Roffo's experiments is of interest, although it must be very rough indeed. Some of Roffo's experiments were confined principally to the winter months, and in those carried out during the summer the animals were not exposed during the hours of most intense insolation.[2] Hence, midday sunlight at the winter solstice can probably be taken as a fair estimate of the average intensity of radiation. Using the data of Pettit (*22*), one may estimate that such sunlight at the latitude of Buenos Aires contains, under ideal conditions, roughly 10^3 ergs per square centimeter per second of effective tumor-producing wavelengths shorter than 3150A. The rats were exposed for 5 hours daily over a period of 7 to 8 months. The total incident energy during this time would be about 4×10^9 ergs per square centimeter, a value somewhat higher than Rusch's. This estimate does not take into account the actual weather conditions, or the difference in distribution of the energy of the mercury arc from that of sunlight. It is only of interest in pointing out that there is no extreme discrepancy between the energies required to produce tumors in rats and mice under two widely different experimental conditions.

SUSCEPTIBILITY

Thus far only rats and mice have been found susceptible to production of tumors by ultraviolet radiation. Abrikosoff and Wail (*28*) describe changes in rabbits' ears resembling basal-cell cancer, as the result of mercury arc radiation, and Rothman and Bernhardt (*29*) report "precancerous" changes; but Rusch (personal communication) has been unable to produce tumors in these animals by prolonged irradiation. Miescher (*27*) did not produce tumors in guinea pigs, but the dosage he used was not sufficient to produce tumors in mice. No experiments on mammals other than rodents have been reported.

Rusch and Baumann (*15*) found that much more radiation was required for tumor production in C57 Brown mice than was required for strain A and strain C mice, both of which are albinos. The difference in this case may be due to the coloration, the pigment of the brown mice serving to filter out the effective radiation.[3]

[1] *This is the energy incident on a flat surface normal to the light rays at the approximate distance of the animals from the source.*

[2] *About half of the animals were lost during the first days of exposure due, apparently, to excessive heating.*

[3] *This evidence should not be carried over to the case of pigmentation in man.* (*See p. 410.*)

GEOGRAPHICAL DISTRIBUTION OF SUNLIGHT AND INCIDENCE OF CUTANEOUS CANCER

There have been numerous claims that skin cancer is more prevalent at regions of the earth which receive greatest insolation. Seldom are these supported by statistics of cancer incidence, and there do not exist sufficient data on the intensity of the tumor-producing wavelengths incident at different regions of the earth to permit a real correlation. The distribution of total sunlight can be estimated with some accuracy, but this is much more difficult for the wavelengths that produce tumors.

Reasons for the difference in distribution of the tumor-producing wavelengths shorter than 3200A as compared with total sunlight, or that portion of it perceived by the eye, can be explained by reference to figure 2. The curve labeled "No atmosphere" in this figure represents the spectral distribution of the sun's radiation as it reaches the outside of the earth's atmosphere [4] while the curve labeled "Midsummer noon Tucson" represents the spectral distribution of the sunlight reaching the earth's surface at noon at the summer solstice at Tucson, Ariz. Comparison of these two curves shows that the intensity for all wavelengths has been materially reduced by passage through the earth's atmosphere, but that the shorter ultraviolet wavelengths which produce tumors (2900A to 3200A) have been reduced much more than the longer visible wavelengths (4000A to 7000A).

The curve, Midsummer noon Tucson, represents maximum insolation reached at that point, the sun being almost at zenith at Tucson at the summer solstice. With any change in season or hour the angle which the sun makes with the zenith increases, with the result that its rays must pass through the earth's atmosphere in a slanting path, which is longer than when the sun is at zenith. In comparing the curves for no atmosphere and at the surface of the earth, it was seen that the proportion of the cancer-producing wavelengths which is absorbed in passing through a given length of the earth's atmosphere is greater than for the visible wavelengths. Hence the difference in the intensity of the radiation from these two spectral regions increases with the length of the path through the atmosphere.

If the length of the path through the atmosphere when the sun is at zenith is taken as unity, the length of the path when the sun is at any other position is numerically equal to sec z, where z is the angle between the sun's position and the zenith, the zenith angle. The term "air mass" is applied to sec z.

Absorption of radiation by the atmosphere follows the Bougeur-Lambert law for absorption in homogeneous media:

$$I = I_0 e^{-kl}$$

where I_0 is the incident energy, I is the energy transmitted by thickness, l, of the absorbing medium, e is the base of natural logarithms, and k is a constant for the given wavelength, the absorption coefficient.

Since sec z measures the thickness of the absorbing layer of the atmosphere, we may write—

$$I = I_0 e^{-k' \sec z}$$

where k' is a constant based on proper units for this absorbing system.

The value of k' is much greater for the short ultraviolet wavelengths than for the visible wavelengths, so that increase in zenith angle affects the former much more than the latter.

The curve labeled "Midwinter noon Tucson," in figure 2 shows the spectral distribution of sunlight at Tucson at the minimum noon insolation of the year, i. e., at winter solstice. At this time the zenith angle is about 54°. Whereas the wavelengths in the visible are appreciably reduced, the tumor-producing wave-

[4] *Obtained by a method of extrapolation from values for incident radiation at the earth's surface, taken when the sun is at different angles to the zenith.* (*See e. g., Pettit* (22).)

lengths are almost completely obliterated. This same spectral distribution would be expected at 54° latitude at noon at the equinox, or at 77° latitude at the summer solstice. While this gives some basis for comparison of the effect of latitude on the incidence of these wavelengths, it does not give a correct picture of their total incidence. The total number of hours of effective radiation increases in the summer as one proceeds toward the pole, and this compensates to a certain extent for the decrease in intensity with latitude. Contour charts given by Kenrick and Ortiz (*30*) illustrate this. The effect is the opposite in winter, of course.

This discussion should give an idea of the complexity of the problem of the relationship between incidence of tumor-producing wavelengths and latitude even under ideal conditions. Actually the problem is still more complex because these particular wave lengths are completely absorbed by a very thin layer of smoke, dust, or other contaminants, whereas this may affect the visible wavelengths very little. On the other hand, the tumor-producing wavelengths are absorbed by water vapor to only a limited extent, so that clouds or fog composed of pure water vapor may permit the major part of this radiation to pass. If, however, the clouds or fog contain slight amounts of smoke or dust, this radiation will be completely removed. These facts account for the apparent capriciousness of sunburn, which is caused by the same wavelengths. It is quite possible, for example, to suffer a severe sunburn on a foggy day provided the fog is not contaminated with smoke or other absorbing material. Likewise, clouds in the open country, or particularly at the seashore, may have little effect in preventing sunburn, whereas in the neighborhood of cities clouds may be contaminated with smoke or dust and hence prevent the sunburn radiation from reaching the earth. Actually, such factors may be far more important than latitude in determining the efficiency of sunlight in producing sunburn and, presumably, cancer of the skin. Altitude may be an important factor, not so much because it reduces the air mass, as that at high altitudes there is less contaminating material in the atmosphere.

The sun's radiation incident on human skin under ordinary conditions is composed not only of rays coming directly from the sun's disk, but includes scattered radiation from the sky. Figure 3 gives measurements of relative intensity of such sky radiation of wavelength 3200A and its distribution with respect to latitude, showing that it may be rather uniformly distributed over the heavens. The relatively small part played by clouds or haze is shown here.

The reflection from the earth's surface itself must vary greatly depending on the nature of the surface in the immediate neighborhood. For example, snow, ice, and water reflect a high proportion of the incident radiation, whereas greensward reflects relatively little. Alpinists in the neighborhood of glaciers or snow fields are often severely sunburned even at relatively high latitudes.

It is thus extremely difficult to estimate the variation of radiation of these wavelengths with respect to geographical position. Sufficient measurements have not been made to permit comparison of the intensities at different parts of the earth, and until such measurements are made, correlation between cancer incidence and geographical distribution of sunlight must be to a great extent a matter of speculation. This statement should not be the basis for too much skepticism, for such a correlation may ultimately be found. If this occurs, and it is definitely established

that sunlight is the determining factor, it is probable that the highest cancer incidence will be found at low latitudes in regions where the atmosphere is particularly clear, but many deviations from a correlation with latitude *per se* must be expected.

Numerous practitioners in Australia (Lawrence (*31*), Molesworth (*32*, *33*), and Duhig (*34*)) describe a high incidence of cancer of the skin and credit this to the aridity of the region, specifically to the high insolation. Only Duhig, however, gives any statistics, namely, that 25 percent of hospital admissions for cancer at Brisbane are cases of skin cancer. The same impression seems to prevail in our own arid Southwest (Smith (*35*)).

Mountin and Dorn (*36*) find the incidence of cancer of the skin three to four times as great in the southern city, Atlanta, as in the northern city, Chicago.

Recently Peller and Souder (*37*), and Peller, Stephenson, and Souder (*38*) have reviewed the occurrence of cancer of the skin and lip [5] in the United States Army and Navy. They find that such cancer occurs much more frequently among soldiers and sailors who were born and presumably have spent their early years in the Southern States than among those born in the Northern States. This is a very interesting correlation, but one must be cautious in its interpretation, since other factors may be involved. One may

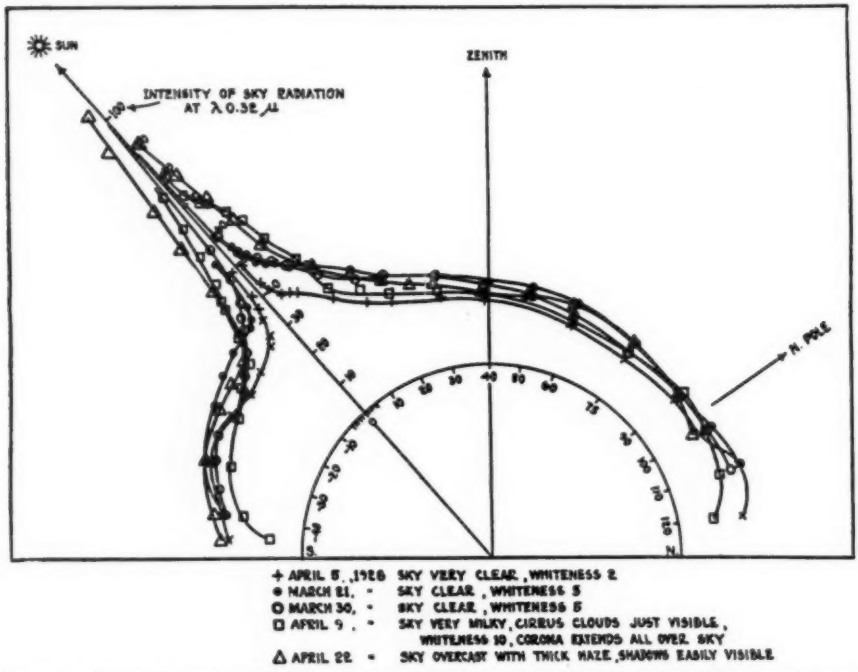
SUN
ZENITH
INTENSITY OF SKY RADIATION AT λ 0.32 μ
N. POLE
+ APRIL 5, 1928 SKY VERY CLEAR, WHITENESS 2
• MARCH 21, " SKY CLEAR, WHITENESS 5
○ MARCH 30, " SKY CLEAR, WHITENESS 5
□ APRIL 9, " SKY VERY MILKY, CIRRUS CLOUDS JUST VISIBLE, WHITENESS 10, CORONA EXTENDS ALL OVER SKY
△ APRIL 22 " SKY OVERCAST WITH THICK HAZE, SHADOWS EASILY VISIBLE

FIGURE 3.—Distribution of radiation of 3200A over the sky at noon near the equinox, at Pasadena, Calif. (Reproduced with permission of Pettit (*25*)).

[5] *Cancer of the lip probably should not be included with cutaneous cancer in this analysis because its etiology can be otherwise explained.* (*See p. 411.*)

question the accuracy of an interpretation of the relatively small difference in latitude represented here, as having a great effect upon the total incidence of the tumor-producing wavelengths in sunlight. However, exposure to sunlight might be much greater in the South because the mildness of the climate permits more time outdoors. Furthermore, a large proportion of the population of the North lives in industrial regions where the tumor-producing portion of sunlight is likely to be greatly reduced by the presence of smoke.

TOPOGRAPHICAL DISTRIBUTION OF CUTANEOUS CANCER AND ITS RELATION TO SUNLIGHT

All authorities agree that cutaneous cancer of human beings occurs most frequently on the face. For example, in 91 percent of the 1,626 cases reviewed by Lacassagne (*39*) the tumors occurred on that area. It has been inferred from this that sunlight is the principal agent responsible for such neoplasms, since the face is probably more exposed to the sun's rays than is any other part of the body. The evidence for this is far from conclusive, and other interpretations may be justified.

DISTRIBUTION OF CANCER OF THE SKIN ON THE FACE

Certain areas of the face are more common sites for cancer than others. This, too, has been interpreted as evidence of the important role of sunlight, the assumption being that the parts of the face receiving most sunlight are those on which cancers are most common. Numerous studies of the distribution of occurrence of cancer on the face have been made, but it is difficult to draw more than very general conclusions from the published data. Where the data are tabulated, the method of dividing the face into regions is not always the same and must be somewhat arbitrary at best. Glasunow (*40*), Magnusson (*41*), and McFarland, Ciccone, and Gelehrter (*42*) express their findings diagrammatically. This has certain advantages. In reporting their data some writers separate cancer of the skin into types whereas others do not, but where this is done it is found that all types are not distributed in the same way. If all skin cancers of the face are considered collectively, it is possible to say that the regions about the nose and the eyes are most frequently affected, but little more can be ventured.

The recognition that the different types of cancer of the skin may have different etiologies, is essential in any attempt to evaluate the importance of a single factor such as sunlight. In the present instance one is almost forced to regard the group as a whole, but the dangers involved in doing this must be realized. It is customary to separate three principal types of cancer of the skin, according to the resemblance of the cancer cells to normal epidermal cell types. Thus are distinguished squamous-cell carcinoma, basal-cell carcinoma, and mixed types. Melanotic tumors and sarcomas are less frequent, and are usually classed separately. Nomenclatures and concepts of histogenesis vary; the reader is referred to Magnusson (*41*) for an introduction to the literature of this subject.

Magnusson (*41*) and McFarland, Ciccone, and Gelehrter (*42*) have mapped the regions of occurrence of cancers belonging to the three groups. Both indicate considerable differences in distribution of the three types, but their data are not in complete agreement. As the latter point out, the separation into types rests on the judgment of the pathologist who makes the microscopic examinations; the distinctions are subtile and provide opportunity for divergence of opinion.

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McFarland, Ciccone, and Gelehrter agree with Glasunow that the basal-cell type of skin cancer occurs chiefly along embryonic lines of closure of the facial skin. This they explain by the presence of rests of embryonic cells in these regions. They find the distribution of squamous-cell, and of mixed-type cancers to be quite different from that of the basal-cell type. If their explanation is correct, one might expect to find corresponding regions of high incidence of basal-cell cancer at other embryonic lines of closure, for example in the median line of the scrotum. McDonagh (*43*) also suggested that the cancers that develop around the nose and eyes develop from embryonic rests but believed that these correspond to remnants of specialized glands in the lower mammals. Magnusson could find no evidence for a correlation between the distribution of cutaneous glands on the face and cancer of the skin, as was suggested by both McDonagh and Lacassagne.

FACTORS DETERMINING EFFECT OF SUNLIGHT ON DIFFERENT AREAS OF THE FACE

The above are examples of hypotheses which assume a purely biological predisposition of certain regions to develop cancer. Lacassagne, on the other hand, follows Dubreuilh (*3*) in the belief that the regions predisposed to cancer are those most affected by sunlight. He believes that squamous-cell and basal-cell cancers arise at different depths in the skin. Like numerous others, e. g., Haythorn (*44*), he believes that the latter occur around hair shafts in the corium. Lacassagne attempts to explain the distribution of the two types of tumor on the basis of penetration of sunlight, assuming that the basal-cell cancers arise only where the epidermis is thin and allows the radiation to penetrate to these deeper layers.

The relative amounts of energy affecting different areas of the face, assuming the same period of exposure, depends upon two principal factors: (1) The intensity of the incident radiation: and (2) its penetration into the skin. The first is determined by the direction of the incident light and the contour of the face. Ordinarily no given area of the face is exposed to direct sunlight for a great portion of the time, and most of the radiation which strikes it must come from the sky. The sky radiation is scattered at all angles, and figure 3 shows that it comes about equally from all points in the heavens. Thus, when the body is erect, all surfaces of the face should receive about the same amount of radiation, with the exception of the under surfaces such as the submental triangle, and areas which are shaded from certain angles such as those behind the ears. The submental triangle seems to be one of the regions least affected in cases of sunburn, and in polymorphic light eruption, a condition of sensitivity to the same wavelength region that produces cancer of the skin in experimental animals (*45*). This was very striking in a case of such photosensitivity that I had opportunity to observe, in which the skin of most of the face exhibited an intense erythematous dermatitis, with the exception of the submental triangle, which was quite pale; there was a sharp line of demarcation between the normal and the affected skin (Blum and Schumacher (*46*)). The submental triangle is also one of the least frequent sites for cancer. It must be the region least often reached by sunlight. It is difficult to go farther in an attempt to analyze the distribution of the incidence of sunlight on the face under ordinary conditions of exposure.

One difficulty arises from the fact that hats or other head coverings may shade portions of the face. Both Magnusson (*41*) in Sweden and Lacassagne in France (*39*)

find tumors quite frequent on the foreheads of women, but rare on this site in men. They account for this on the assumption that men habitually wear hats while women do not. On the other hand, they find tumors of the ear quite common in men, but rare in women. This these writers credit to protection of the women's ears by shawls. Magnusson indicates about the same distribution for senile keratoses, which are generally considered to be precancerous manifestations. He reproduces two quite convincing photographs, one of a man with cancer of the ear and senile keratoses of the exposed areas of the face the skin of which is quite normal in the forehead area ordinarily protected by a hat, and another of a woman with senile keratoses of the face with the exception of the ears and an area of the cheek just below corresponding to the region habitually covered by a shawl. Dubreuilh (*3*) also points out instances of precancerous skin and cancer occurring strictly on regions not covered by the particular head clothing worn by the patient.

Habits and mode of dress vary from country to country and from period to period, and this variation makes it difficult to estimate the value of such conclusions when applied to different sets of data. It is interesting on this account to find that the data of Heiman (*47*) (1899), of Berlin, Borrmann (*48*) (1905), of Göttingen, and Meller (*49*) (1907), of Vienna, confirm those of Lacassagne, of Paris, and Magnusson, of Stockholm, as regards the preponderance of tumors of the forehead in women. Meller's data indicate more ear tumors in men, but Heiman's do not, and Borrmann's indicate greater incidence in certain regions other than the ear. [6] In contrast to the above impression as regards the distribution of head clothing between the sexes, Taussig and Williams (*5*) (1940), of St. Louis, think that the forehead of women is less exposed to sunlight than is that area in men, and this may well be true in the United States at this time. No data on the distribution of cancers of the face as regards the distribution between the sexes seem to have been published from the United States.

Penetration of the skin has been mentioned above as an important factor in determining the effectiveness of sunlight. The wavelengths which produce tumors in rats and mice and sunburn and antirachitic action in man, that is, those shorter than 3200A, are completely absorbed within half a millimeter or less of the surface. Only a slight amount penetrates deeper than the epidermis, depth of penetration being different for different wavelengths. Figure 1 shows the spectral transmission of human epidermis as measured by Lucas (*23*). According to these measurements, wavelength 3000A is 44 percent transmitted by epidermis 0.08 mm. thick, but less than 5 percent by wavelengths 2900A and shorter. Bachem and Reed (*51*) found lower values than Lucas—about 5-percent transmission at 3000A for the same thickness of epidermis. Such measurements are subject to considerable errors which derive principally from the scattering of the radiation in the epidermis. (See below.) Lucas' and Bachem and Reed's measurements were obtained with different methods, and it is possible that the differences in their values may be explained, at least in part, by differences in the effect of scattering on the measurements.

Let us analyze the importance of these findings as regards sunburn [7] of normal

[6] *All data agree that tumor of the lower lip is predominant in man.* (*See p. 411.*)

[7] *This term will apply herein to the erythema and subsequent tanning caused by radiation of 3200A and shorter wavelengths, regardless of the degree of these changes.* (*See p. 412.*)

skin. The primary changes of sunburn take place principally in the prickle-cell layer, according to the evidence of Guillaume (*52*) and Keller (*53*). This layer lies beneath the corneum and granulosum which do not take part in the response, but act only as filters to cut off a part of the incident radiation. The thickness of these layers is thus very important in determining the effectiveness of the radiation which strikes the surface of the skin. In fact, a great deal of the decrease in sensitivity of skin to sunlight that results from repeated exposure is due to increase in thickness of the corneum and granulosum, attendant upon proliferation of the stratum germinativum. Miescher (*54*) has shown that the corneum may thicken as much as five or six times, as a result of exposure to ultraviolet radiation. It is commonly thought that such decrease in sensitivity to sunlight is due to pigmentation which accompanies these changes. Actually, in the white races, most of the pigment is too deep to be of great importance in this respect, although it may protect the deeper layers. (See p. 410.)

It is obvious from this that the thickness of the epidermis, particularly the corneum, on different parts of the face might be of importance in determining the location of skin cancer if sunlight is an important etiologic factor. Magnusson examined this, however, and found the difference in the thickness of the epidermis from one part to another to be insignificant. The values ranged from 0.032 mm. on the forehead and temple to 0.042 mm. on the upper edge of the ear. Thus, penetration of the radiation should be so nearly equal on different parts of the face as to have little significance in determining the distribution of tumors. No support for Lacassagne's hypothesis is found here.

In attempting to analyze this question, Magnusson has used the following equation, which does not take into account a number of important factors

$$W=\frac{t}{\sin a} \tag{3}$$

where W is the length of the pathway passed through to reach the basal-cell layer of the epidermis, t the thickness of the epidermis, and a the angle the incident radiation makes with the skin surface.

In general, radiation entering an absorbing system is both absorbed and scattered. In the epidermis a great proportion of the radiation is scattered as the measurements of Lucas (*23*) show. Absorption follows the Bougeur-Lambert law (*1, p. 13*), and scattering follows a similar relationship as long as it is uniform throughout the system. We may therefore write

$$I=I_o e^{-KI} \tag{4}$$

where K includes constants for both absorption and scattering. K is known as the attenuation coefficient. Bachem's (*55*) measurements show that attenuation of these wavelengths by the epidermis follows such a relationship approximately.

The incident intensity for direct sunlight (in which the rays are parallel) varies as the cosine of the angle which the light rays make with the normal to the skin surface, i. e.,

$$I_i=I_s \cos \theta \tag{5}$$

where I_i is the incident intensity, I_s is the intensity of the sunlight, and θ is the angle of incidence.

A few percent of I_i is reflected from the skin (Luckiesh, Holladay, and Taylor (*56*) and Schultze (*57*)), but most of it enters the epidermis as I_o. If as stated earlier, the skin is ordinarily subject to radiation from all angles, it is reasonable for purposes of analysis to consider the incident radiation as a mean value $\bar{I}_i$ for a ray striking at normal incidence. I_o is then a definite large fraction of $\bar{I}_i$.

Since the radiation is almost completely scattered, the direction of the incident rays has no significant effect on the direction of the rays within the skin. With these considerations in mind we may apply equation (4) to the problem of penetration of sunlight into the epidermis.

Even though the values of K for epidermis are rather high, the difference in the intensity of radiation reaching the lower surface would not be greatly different for the two extremes of epidermal thickness for the face, measured by Magnusson—0.032 mm. and 0.042 mm. The corresponding amounts of radiation of wavelength 3000A reaching the basal-cell layer at the bottom of epidermis of these thicknesses would be respectively 65 and

72 percent, if Lucas' values are used for calculating K, and 20 and 30 percent if Bachem's values are used. In either case the differences are not great enough to permit any correlation to be drawn between the penetration of such radiation and the distribution of cancer of the face, as was proposed by Lacassagne.

FACTORS OTHER THAN SUNLIGHT AFFECTING THE SKIN OF EXPOSED PARTS

It is obviously impossible with our present knowledge to explain the distribution of cancer on the face in terms of differences in effectiveness of the sunlight striking the skin; and unless more accurate measurements can be produced, one is forced to conclude that certain regions are more susceptible to such neoplasm than others, no matter what the inciting agent may be. Explanations based on the embryonic rest hypothesis have already been discussed, and it may be profitable to consider other factors that might account for the distribution.

Haxthausen (*58*) has pointed out that certain skin diseases have very definite sites of preference. Some, for example, lupus erythematosus, follow a distribution not unlike cancer of the skin (i. e., the region about the nose and eyes is most frequently affected). Haxthausen emphasizes the fact that all exposed areas tend to undergo permanent dilatation of the minute skin vessels due to the action of sunlight and of cold, and that this is particularly true for the areas mentioned. Without attempting to evaluate the importance of these two agents, or of other environmental factors that affect only the exposed parts, it must be pointed out that those parts enjoy a different physiological state than the covered portions of the body, and that this difference increases with the age and exposure of the individual. This is reflected in other chronic changes in the exposed skin of persons of advanced years, where besides the diffuse reddening due to vascular dilation, are found degeneration of the collagen and elastic tissues, and a tendency to deposit pigment in large patches (Hausmann and Haxthausen (*59*)).

It must also be remembered that the face and hands may more frequently undergo minor trauma than unexposed parts of the body. For example, frostbite has been credited with causing cancer (Magnusson (*41*)). These parts also come in contact with dust and other materials that may contain chemical carcinogens. This is certainly important among industrial workers handling coal tar and its derivatives, and contact with dust containing carcinogens may occur in other industries.

THE CHARACTER OF THE SKIN, AND CANCER

BLONDS AND BRUNETS

"A commonplace in dermatologic lore is that skin cancer occurs more frequently in blonds than in brunets." This statement prefaces a recent article of Taussig and Williams (*50*). Certainly such a correlation is frequently mentioned (McCoy (*60*), Molesworth (*32*), Roffo (*61*)), but the terms "blond" and "brunet" are difficult to define and may have different meaning to different observers. Taussig and Williams, realizing this difficulty, have devised quantitative measurements which indicate that certain characteristics of skin color are associated with skin cancer. The results are interesting and no doubt significant, but it may be too early to evaluate them.

Here it will only be possible to discuss some of the factors determining skin color that are of particular interest as regards the action of sunlight on skin. Skin color depends principally upon two factors, the amount and color of the blood in the minute vessels of the skin and the amount of melanin pigment in the epidermis.

The first factor must depend on the vascularity of the skin and upon physiological factors which determine the degree of oxidation of the blood. Vascularity of the skin may be of a permanent nature as exemplified by the increased redness of exposed areas mentioned above. (See p. 409.) This is subject to transitory physiological changes either local or systemic, as is also the color of the circulating blood.

The amount of pigment and its color are determined not only by hereditary differences, but by the amount of exposure to ultraviolet radiation. Ultraviolet radiation of wavelengths shorter than 3200A brings about increase in the amount of pigment in the epidermis. The essential mechanism involved is probably the same as that which produces erythema, to which pigmentation is a normal sequence (Blum (*45*)). Apparently the pigment can be present in a colorless form, and it has been recently shown by Henschke and Schultze (*62, 63*) and Miescher and Minder (*64*) that wavelengths including the region from about 3000A to 4000A cause darkening of this preformed pigment. (See p. 413.)

The location of the pigment in the epidermis is of particular interest with regard to the penetration of radiation. In the white races it is found principally in the basal-cell layer as granules. From here it migrates superficially and becomes more diffuse. It is commonly believed that the pigment which appears in the skin after exposure to ultraviolet radiation serves as a light filter, thus rendering the skin less sensitive to subsequent exposure to such radiation. The pigment appears in the basal-cell layer beneath the region in which the primary changes of sunburn occur (p. 413), and it is questionable that it can afford much protection in sunburn (*65*). Certainly the experiments of Miescher (*54*) show that a great deal of the reduction in susceptibility to ultraviolet radiation is due to thickening of the corneum. (See p. 408.) Hence, it is somewhat doubtful that the intensity of pigmentation is a trustworthy index of susceptibility to ultraviolet radiation. On the other hand, Ellinger (*66*) finds that blonds are as a whole more susceptible to such radiation than brunets.

It was pointed out above that the reddening of the skin due to vascular changes may result from exposure to changes in temperature, or to other factors as well as sunlight, and hence caution must be used in correlating skin color with the extent of exposure to sunlight. All these considerations point out the danger of reaching conclusions a priori regarding skin color and susceptibility to sunlight.

What has been said about the function of pigment in protecting against the penetration of sunlight applies only to the white races. In Negroes the pigment is not only more plentiful, but is distributed throughout the epidermis, thus providing important protection against sunburn (*65*). It is, therefore, highly suggestive that cancer of the skin is rare among Negroes. Schamberg (*67*) states that he has seen only one case of cancer of the skin in a Negro (a mulatto) although he has treated thousands of cases of this disease. Of 178 cases of cancer of the skin examined by Hazen (*68*) only 4 were Negroes. This immunity seems to extend to other dark-skinned races. Lain (*69*) states that he has never seen skin cancer in an American Indian, and Roffo (*61*) reports from Argentina that all his cases of skin cancer were in foreigners or immigrant families, none of Indian or Negro blood. The incidence was much lower in families established in that country for several generations than in newcomers.

XERODERMA PIGMENTOSUM

Xeroderma pigmentosum is an inherited condition characterized by skin changes,

among which freckling is an early expression. Neoplastic changes ultimately develop, and the individuals so affected usually die before adulthood. This condition is commonly thought to result from exposure to sunlight, but sunlight is probably an exacerbating factor rather than a fundamental cause (*45*). The disease is rare in occurrence, being inherited as a recessive character (*70*). There is evidence, however, that it is only partially recessive, since some members of the families in which the disease occurs display a degree of freckling uncommon in other than reddish blonds where freckling is often characteristic. This group of individuals may include certain cases called "xeroderma pigmentosum tardivum," which resemble xeroderma pigmentosum but occur in later life. There is a suggestion here of an inherited tendency toward skin malignancy in apparently normal individuals which might be set off by exposure to sunlight. Molesworth (*32*) describes a family of eight brothers, all outdoor workers, of whom six developed cancer of the face. Such considerations make important the study of hereditary relationships in cancer of the skin.

OCCUPATION AND CUTANEOUS CANCER

It is commonly believed that cancer of the skin occurs more frequently in outdoor workers than in those occupied indoors. This is reflected by such terms as seaman's skin, peasant's skin, and farmer's skin, applied to allegedly precancerous changes when they occur on the exposed parts of persons of the corresponding occupations. Sunlight is generally credited with bringing about these changes although, as has been mentioned, there may be other causes.

It is obviously difficult to obtain statistics on this. Perhaps the most convincing are those of Peller and Stephenson (*71*), who point out that mortality from cancer of the skin and lip (see below) is about three times as high for the United States Navy as for the average population of the same age group. On the other hand, Magnusson (*41*) points out that in Sweden the percentage of farmers with cancer of the skin is about the same as the percentage of farmers in the population. Possibly sunlight is not an important factor at this high latitude. Young and Russell (*72*) find a predominance of cancer of the skin among sailors, and particularly those who work on deck. They credit this principally to contact with chemical carcinogens, however, pointing out that such sailors handle tarred ropes and wear oil-treated clothing.

Alleged predominance of cancer of the skin in men has been credited to greater outdoor activity of that sex, and correspondingly greater exposure to sunlight. The statistical data on this point are somewhat confused. Some writers include cancer of the lip with cancer of the skin, whereas others do not. There seems no doubt that cancer of the lip occurs much more frequently in men than in women, and since this is allegedly due to factors having nothing to do with sunlight, it seems best not to include it with cutaneous cancer when considering the etiologic role of sunlight. The data of Heiman (*47*), Borrmann (*48*), Meller (*49*), Lacassagne (*39*), and Mountin and Dorn (*36*) indicate a preponderance in the male sex if cancer of the lip is excluded. The difference between the sexes is very small in some instances, however.

THE MECHANISM OF CUTANEOUS TUMOR PRODUCTION

In examining the evidence that sunlight is an etiologic factor in cancer of the skin in man, it has been assumed that the

wavelengths involved are the same as those that cause tumor formation in mice and rats. Some may object to this extrapolation, but it can be made with as much assurance as most extrapolations from laboratory animals to man that are commonly accepted. Lacking more direct evidence we are justified in adopting it as the only basis for discussion. In the following pages it will become evident that this basic assumption is reasonable, provided quantitative differences are recognized.

MECHANISM OF PHOTOBIOLOGICAL EFFECTS IN GENERAL

So far as photochemical effects in living systems are concerned, radiation of different wavelengths may act very differently; not so much through virtue of difference in the radiation itself, as due to the fact that different substances within the living system absorb different wavelengths. In order for photochemical reaction to take place some substance in the system must be activated by absorption of radiant energy, and hence only wavelengths absorbed by that substance can forward the reaction. This substance will be referred to for convenience as the light absorber.

Thus, the wavelengths that produce a given photobiological process must be limited to those absorbed by the light absorber. It might be expected, moreover, that the relative effectiveness of the various wavelengths (called the action spectrum (fig. 1)), would follow closely the absorption spectrum of the light absorber. Under favorable conditions, if absorption is expressed in the proper units, very close agreement between action and absorption spectrum may be demonstrated. More frequently close agreement is not found, however, but ordinarily the action spectrum shows some resemblance to the absorption spectrum. The writer has discussed elsewhere (*45*) the conditions necessary for close agreement and the numerous factors which may cause deviations under actual conditions.

The character of the action spectrum may be the clue to the nature of the absorbing substance, and it is one of the first things that should be determined in the study of a photobiological process. In the case of tumor production, only the general limits of the action spectrum are thus far known. (See p. 398.) These limits fall within the same spectral region as the action spectra of known photoprocesses in human skin, and hence a possible relationship to those processes must be considered.

THE ACTION OF RADIATION ON HUMAN SKIN

Three independent photoprocesses in normal human skin are known to be brought about by wavelengths included in sunlight.[9]

The first will be referred to as the sunburn mechanism. It comprises a series of events, all of which result from damage to the stratum germinativum of the epidermis, principally the prickle cells (Keller (*53*)). The first observable manifestation is erythema which represents dilatation of the vessels of the papillary layer. This usually appears an hour or so after exposure to the radiation. It is followed by pigmentation, i. e., the appearance of melanin pigment in the epidermis of the irradiated area.[10] The action spectrum appears as curve E in figure 1.

The second photoprocess, antirachitic action, seems to be a photochemical reaction by which 7-dehydrocholesterol or some closely related sterol compound

[9] *Certain other effects may be brought about under artificial or disease conditions, but these cannot be regarded as normal skin responses (45).*

[10] *It must be pointed out that only the erythema produced by ultraviolet radiation is considered herein. This discussion does not apply to erythema produced by X-rays, which seems to be qualitatively different in mechanism.*

is changed into vitamin D or another antirachitic substance. The action spectrum appears as curve A in figure 1.

The third process, only recently described with accuracy, is darkening of pigment already present in a colorless form (Henschke and Schultze (*63*), Miescher and Minder (*64*)). This process requires about one thousand times as much energy as the sunburn mechanism. Its action spectrum lies between 3000A and 4000A and therefore does not correspond with that for tumor production. It is doubtful, therefore, that it has any role in this process, and it will not be further considered here.

The first two processes, on the other hand, have approximately the same long wavelength limit as tumor production, i. e., 3200A, and must therefore be examined as to their possible involvement in that phenomenon.

THE SUNBURN MECHANISM AND CANCER

Numerous hypotheses have been proposed to explain the erythema and pigmentation of sunburn. The only concept that seems to fit all the evidence is that both are sequels to injury to the cells of the stratum germinativum, principally the prickle cells, brought about by the action of ultraviolet radiation. Apparently the corium is not primarily affected in sunburn although secondary changes occur there. (See Blum (*45*) for a a discussion of alternative hypotheses.) It seems probable that the point of attack, i. e., the light absorber, is the protein of these cells, which absorbs strongly in the region of the sunburn producing wavelengths but does not absorb longer wavelengths. All proteins containing benzenoid rings have absorption maxima at about 2800A where there is a minimum in the action spectrum for erythema; but this may be accounted for as due to the filtering action of the corneum, which does not take part in the reaction. The corneum, like the rest of the epidermis, has an absorption maximum at about 2800A (fig. 1) which must tend to shift the maximum of the action spectrum toward longer wavelengths. Mitchell (*73*) shows that the position of the erythema maximum can be accounted for in this way if it is assumed that the absorbing substance is a typical protein.

Rusch's experiments indicate that the action spectrum for cancer production does not agree with the erythema spectrum, since he could produce no tumors with radiation of wavelength 2537A although this is quite effective in producing erythema (table 1, and fig. 1). In sunburn the primary change is in the epidermis, whereas for cancer production it may be necessary to affect deeper layers also, i. e., the corium. In this case the epidermis as a whole must be regarded as a filter, and reference to figure 1 will show that the action spectrum might be restricted virtually to only those wavelengths longer than about 2800A. Such an explanation receives some support from the fact that sarcomas form a considerable part of the tumors produced by ultraviolet radiation in the laboratory (Roffo found 36 percent sarcomas), but caution should be used at this point in carrying over the evidence from experiments on rats and mice to the problem of cancer in man. The epidermis of rodents is much thinner than that of man, at least before the former has been subjected to ultraviolet radiation. The erythema action spectrum for these animals has not been accurately determined, although it is known to have the same long wavelength limit as in man.

So far as the evidence from action spectra goes, then, it is quite possible that cancer production is the result of photochemical changes similar to those causing the ery-

thema of sunburn, but occurring in cells at a deeper layer.

Mitchell (*73*) has put forward the hypothesis that the ultraviolet radiation acts directly upon the proteins of the cells of the stratum germinativum to form breakdown products of high molecular weight that diffuse to the vessels of the papillary layer, and cause the dilatation which is observed as erythema. He would involve these same vasodilator substances in the etiology of cancer. He attempts to explain the long latent period of erythema production as due to the time required for these vasodilator substances of high molecular weight to diffuse to the vessels. However, Clark (*74*) has shown that the latent period of erythema has a high temperature coefficient, which would not be compatible with a diffusion process; and it seems much more probable that the latent period represents the time required for the elaboration of a dilator substance by the cells. This must be governed by a thermal reaction since it has a high temperature coefficient. On the other hand the initial part of the reaction is independent of temperature as the dosage of energy required to produce erythema is not affected by temperature (*74*). This is characteristic of photochemical reactions. Mitchell's hypothesis also encounters difficulty in the fact that the action spectrum for tumor production does not agree with that for erythema.

STEROLS AND CANCER

There is no reason to believe that the antirachitic mechanism is involved in cancer production. It has not been demonstrated that antirachitic substances or their precursors are carcinogens (Bachmann et al. (*75*)). However, similarity in chemical structure to some of the carcinogenic hydrocarbons has suggested that such carcinogens may result from the irradiation of these or other sterols in the skin.

There is general agreement that increase of skin cholesterol results from ultraviolet radiation. Kawaguchi (*76*) found such an increase in guinea pigs, and Roffo (*77*, *11*) and Knudson, Sturges, and Bryan (*78*) in rats. Baumann and Rusch (*79*), on the other hand, found this increase in rats, but not in guinea pigs. Roffo (*80*) states that increase of cholesterol in human skin is produced by mercury arc radiation. He also states (*17*, *77*) that the cholesterol content is greatest in those regions of the skin most exposed to sunlight, which are likewise the most common sites for cancer. Knudson, Sturges, and Bryan find an unequal distribution of cholesterol in different areas of the skin of rats, which parallels this.

Cholesterol itself is not a carcinogen (*81*) and although its accumulation in irradiated skin is an indication of changes taking place there, this alone tells very little about the mechanism of cancer production. Roffo (*61*) suggests that the cholesterol is altered by the radiation. His evidence on this point seems, however, to be refuted by the observations of Lucas (*82*), Mayneord and Roe (*83*) and Stavely and Bergmann (*84*). Bergmann et al. (*85*, *86*) have made numerous unsuccessful attempts to produce carcinogenic substances by irradiating cholesterol. Other evidence against the concept that irradiation products of cholesterol are responsible for tumor production is that of Wahlgren (*87*) and Baumann and Rusch (*79*) who could not increase the incidence of tumors by feeding that substance to animals that were exposed to mercury arc radiation. Rusch, Baumann, and Kline (*88*) also painted animals with cholesterol during the course of such radiation. They found tumor production was not affected by the cholesterol although in some cases

the solvent for the cholesterol enhanced that effect. They believe this was due to increased penetration of the radiation caused by the solvent. This seems highly probable, since such substances may cut down the scattering of the radiation in the epidermis and thus allow greater penetration. (See p. 408.)

While the idea that sterols in the skin may be changed to carcinogens by the action of ultraviolet radiation is an attractive one, it must be admitted that there is little evidence in its favor at present.

PHOTOSENSITIZATION

It has recently been found that polycyclic carcinogenic hydrocarbons may sensitize living systems to light. This has led to speculation as to the participation of such substances in the production of cancer by sunlight. That these compounds play a fundamental role as absorbers of radiation in that process seems unlikely, however, when action and absorption spectra are considered.

These carcinogenic hydrocarbons absorb wavelengths longer than 3200A to a considerable extent (e. g., Mayneord and Roe (*89*)), and hence the action spectrum for photosensitization by these substances should extend to longer wavelengths. This is borne out by the experiments of Doniach and Mottram (*90*), who found that benzpyrene and dibenzanthracene sensitized mice to "blue and violet," and (*91*) paramecia to 3500A to 4100A. These measurements seem to disagree, but they were obtained with filters, and the limits may not be very exact. The values 3500A to 4100A for paramecia agree quite well with an important region of absorption by these hydrocarbons. At any rate, the action spectra extend to wavelengths longer than 3200A, and if such reactions were fundamental to tumor production, such wavelengths should be active in that process. This is not the case, the action spectrum for tumor production having its long wavelength limit at 3200A.

The discovery of the photosensitizing action of the carcinogenic hydrocarbons has led to the hypothesis that light accelerates their carcinogenic action. This seems improbable. The ability to sensitize living systems to light is displayed by a very large number of substances whose chemical formulae have little or nothing in common. The group includes fluorescein dyes, porphyrins, acridine dyes, and many fluorescent compounds other than the carcinogenic hydrocarbons. Such photosensitization of living systems, the results of which are usually destructive, has been given the name photodynamic action.

This phenomenon finds complete analogy in certain photosensitized oxidations *in vitro*. These are oxidations of appropriate substances by O_2, which are activated by energy absorbed by the photosensitizer. The photosensitizer captures a quantum of radiant energy and immediately transfers the energy so obtained to some molecule which takes part in the oxidation, probably the substance that is oxidized. The photosensitizer molecule itself is not changed chemically, but returns to its original state after giving up its quantum of energy, and can then absorb and transfer another quantum. Thus it may participate repeatedly in the reaction, bringing about the oxidation of many molecules of substrate without being itself altered.

The ability to accomplish this would appear to reside in a property of the molecule that permits it to retain an absorbed quantum for a finite activation period (usually from 10^{-9} to 10^{-6} seconds). Within this time it may transfer the energy to another molecule which is able to receive it. This property of the molecule is the same that permits it to fluoresce,

fluorescence being the act of reradiating an absorbed quantum within the activated lifetime of the molecule. Thus, it would seem that all fluorescent substances are potentially capable of photosensitizing such oxidations, subject to numerous restrictions.

Photodynamic action is undoubtedly this type of photosensitized oxidation taking place in living systems. Photodynamic action always requires O_2, but differs strikingly from normal O_2 metabolism in a number of ways. The photochemical characteristics of photodynamic action resemble in all ways those of the photosensitized oxidations, including repeated participation of the photosensitizer molecule in the reaction (Blum and Gilbert (*92*)). By analogy it is to be expected that any fluorescent substance may act as the light absorber for photodynamic action, but there is at least one important restriction, in that the fluorescent substance must be taken up by the cell. The apparent photodynamic effectiveness of a fluorescent substance seems to rest principally on the latter property. The evidence on which the above description is based is reviewed elsewhere by the writer (*45*).

There is nothing to distinguish the photosensitization of living systems by the carcinogenic hydrocarbons, from photodynamic action; and in at least one instance the requirement of O_2, which is a fundamental characteristic, has been demonstrated (*45, p. 66*). Hence there is no reason to predict that light has any effect on the carcinogenic action of these compounds, since the ability to produce photodynamic action is not directly related to the chemical properties of a substance.

Various investigators have carried out experiments to test the effect of light on the incidence of cancer produced by chemical carcinogens, by exposing animals painted with these substances to radiation from various sources. Some of these are summarized in table 2. There is little agreement of results, but differences in experimental procedure may account for this. In some cases the cancer-producing wavelengths are eliminated, so that whatever effect occurs must be due to some reaction in which the carcinogen takes part. In other instances the effect of wavelengths shorter than 3200A would seem to be superimposed upon that of the carcinogen itself. One might expect an addition of effects in such cases, but it seems probable that in most instances the carcinogen on the surface of the skin serves as a filter which reduces the intensity of the cancer-producing wavelengths. The experiments of Morton, Luce-Clausen, and Mahoney (*98*) are perhaps the most interesting of the group. In these the cancer-producing wavelengths were eliminated. The animals displayed symp-

TABLE 2.—*Effect of light on tumor production in mice painted with chemical carcinogens, by exposure to radiation from various sources*

Investigators	Carcinogenic substance	Source of radiation	Wavelengths shorter than 3200A included	Effect on tumor production
Findlay (*9*)	Tar	Hg arc	Yes	Increased.
Kohn-Speyer (*93*)	do	do	Yes	No effect.
Seelig and Cooper (*94*)	do	Diffuse daylight	No	Do.
Maizin and DeJonghe (*95*)	Benzpyrene	do	No	Increased.
Vlès, De Coulon, and Ugo (*96*)	Tar	Filament lamps	No	Do.
Taussig, Cooper, and Seelig (*97*)	Benzpyrene [1]	SI lamp	Yes	No effect.
Rusch, Baumann, and Kline (*88*)	1, 2,5,6-Dibenzanthracene	Hg arc	Yes	Do.
Morton, Luce-Clausen, and Mahoney (*98*)	3,4-Benzpyrene	Fluorescent lamp	No	Decreased.

[1] Some mice also injected with hematoporphyrin.

toms of photosensitivity, showing that the light was intense enough to have some action. The authors are inclined to credit the decreased tumor incidence under these conditions to the fact that the photosensitized animals were sick, and less subject to development of tumors. It is also possible that the concentration of the carcinogen was reduced by photooxidation.

It has been suggested that the carcinogens are rendered more active by changes in their chemical structure brought about by light. Photosensitizers themselves may undergo photooxidation if there is no oxidizable substrate present. In some instances these oxidation products are more destructive to living systems than are the parent substances, but this is not the basis for photodynamic action (*45*). Rideal (*99*) has found that previously irradiated carcinogenic hydrocarbons are very active in destroying protein films, but this is also true for fluorescein dyes (*45*). This is of questionable importance as regards carcinogenesis, since it is doubtful that this photooxidation would take place in living systems where other oxidizable material is plentiful.

Some substances that cause photodynamic action may participate in photochemical reactions other than oxidations by O_2. Characteristically they may act as hydrogen acceptors, thus bringing about dehydrogenation of a proper substrate, but only when O_2 is not present. It is improbable that such reactions could occur in the skin, which is well supplied with oxygen under most circumstances.

Büngeler (*100*) carried out experiments with mice which appear to show that photodynamic action can enhance tumor formation by sunlight. He used substances which in the absence of light are not carcinogens, i. e. eosin and hematoporphyrin. A coal-tar preparation, Anthrasol, produced about the same incidence of tumors under these conditions. The writer has carried out limited experiments on rats, using rose bengal as the sensitizer and excluding wavelengths shorter than 3200A, but produced no tumors. The dosage of light and photosensitizer was sufficient to produce severe skin lesions. Porphyrins have been invoked as casual agents in almost all types of photobiological process, but their participation in any one, other than artificially produced photodynamic action, has yet to be clearly demonstrated (*45*). Körbler (*101*) has suggested their role in the production of human cutaneous cancer, but this seems to be ruled out, since these compounds sensitize to wavelengths longer than 3200A.

PHOTOSENSITIZATION OF WORKERS WITH COAL TAR

Workers with coal-tar derivatives sometimes become sensitive to light (Kistiakowski (*102*) Foerster and Schwartz (*103, 104*)), and this has been connected with the occurrence of cancer of the skin among such workers. On the basis of what has been said above, it seems most probable that these phenomena are independent. The occurrence of cancer is no doubt due to the presence of carcinogenic hydrocarbons. The photosensitization, on the other hand, may be in part due to these substances, but coal tar contains other photosensitizing substances, e. g., anthracenes and acridines. The wavelengths responsible for this photosensitization lie in the longer ultraviolet and shorter visible according to Foerster and Schwartz.

CONCLUSIONS

Converging evidence from a number of sources indicates sunlight as an important etiologic factor in cutaneous cancer of human beings. However, the evidence is tenuous in most instances. The clinical

evidence alone is suggestive, but that from animal experiments is necessary to lend solidity to the concept. Caution is necessary, however, in carrying over the evidence from these experiments to the case of man.

The determination of the wavelengths that produce tumors in experimental animals is a very important step in the solution of the problem. Since these are the same wavelengths that principally effect human skin, i. e., those shorter than 3200A, it seems probable that they, if any, play a role in human cutaneous cancer. Numerous tentative hypotheses regarding the mechanism of tumor production by radiation seem untenable because they require the participation of longer wavelengths.

Much more exact data, both clinical and experimental, are required to place the concept on a thoroughly sound footing.

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